THE IMMUNOLOGY OF INFLUENZA: FROM PATHOGENESIS TO VACCINES

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ABSTRACT

Influenza is a respiratory pathogen that, despite the availability of vaccines, continues to have an enormous impact on human health and world economy. In addition to seasonal epidemics, the persistent threat posed by newly emergent pathogenic strains highlights the need to better understand mechanisms that underlie protective and pathogenic host responses. To address this issue, we used a multi-pronged approach. First, we sought to examine humoral and cellular immunity against seasonal and novel influenza viruses using a human vaccine cohort established during the 2006-07 and the 2007-08 influenza seasons. The vast majority of past studies have focused on strain-specific serum antibodies as the sole correlate of protection against influenza. However, we show that in addition to antibody responses, seasonal trivalent inactivated (TIV) and live attenuated (LAIV) influenza vaccines also induced significant increases in influenza-specific T cells post vaccination. Importantly, unlike antibodies, T cells established by seasonal vaccination and/or infection were highly cross-reactive against novel influenza viruses, namely the pandemic 2009 H1N1 virus. In addition, we also detected significant and sustained changes in serum cytokine profiles after vaccination with TIV but not LAIV suggesting that the different vaccines may activate different immunological compartments. Future studies examining mucosal immune responses may provide more insight into immunological mechanisms underlying protective immunity following LAIV. In the second part of this thesis, we examined the role of the potent anti-inflammatory cytokine IL-10 in the pathogenesis of lethal influenza infections using a mouse model. IL-10 deficiency was, somewhat counterintuitively, associated with significantly lower morbidity and mortality suggesting that expression of IL-10 was pathogenic during lethal influenza infections. IL-10 expression in WT animals was associated with higher pulmonary damage and compromised pulmonary function compared to *Il10*$^{-/-}$animals. Interestingly, IL-10 negatively regulated the

expression of IL-22, a cytokine that has recently emerged as an important mediator of tissue homeostasis, particularly in the mucosa. However, improved disease outcomes in *Il10*$^{-/-}$ was independent of IL-22 as genetic ablation of IL-22 in *Il10*$^{-/-}$ mice (ie. *Il10*$^{-/-}$*Il22*$^{-/-}$) had no impact on survival rates. Finally, using a less virulent strain of influenza, namely A/WSN/33, we show that the pathogenic effects of IL-10 may be strain- specific. Considering the enormous toll on human health, the development of more effective vaccines and therapeutics is a public health priority. A better understanding of host factors that mediate protective immunity as well as drive pulmonary damage is undoubtedly an important step in the rational design of next generation therapeutics. The data presented in this dissertation serves to underscore the importance of mouse models and human vaccine cohorts as important vehicles in this process.

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Thesis Advisor:	Dr. Jay H. Bream
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CHAPTER 1: Introduction

1.1 Influenza: Epidemiology, virology and clinical features

Influenza is a globally important respiratory pathogen that continues to pose a significant public health problem. Infecting between 10-20% of the world's population every year, influenza is one the leading causes of infectious respiratory disease today (1,2). Seasonal infections result in 3 to 5 million cases of severe disease worldwide (World Health Organization WHO factsheet 211: influenza; 2003) and between 17,000-51,000 deaths in the United States every year (3). The annual economic burden associated with these recurrent infections and hospitalizations is estimated to be a staggering $87 billion, with a majority of this burden borne by young children and individuals over the age of 65 (4). In temperate zones, annual epidemics tend to peak during the winter seasons, while in tropical regions infections can occur throughout the year. The exact cause for this seasonality is not clear.

Influenza viruses are unique in terms of their ability to cause recurrent annual epidemics as well as more serious pandemics. Due to low pre-existing immunity to newly emergent influenza strains, pandemic influenza viruses have the capacity to infect a much larger proportion of individuals and cause morbidity and mortality on a global scale. It is estimated that the 1918 pandemic "Spanish flu" resulted in the death of greater than 50 million people across the world with over 500,000 deaths in the United States alone (5). There have been three subsequent pandemic in the last century including the 1957 "Asian flu", 1968 "Hong Kong flu" and 2009 swine-origin H1N1. Continued human infections with highly pathogenic avian influenza strains such as H5N1 and H7N9 virus underscores why efforts to develop new vaccines and antiviral agents

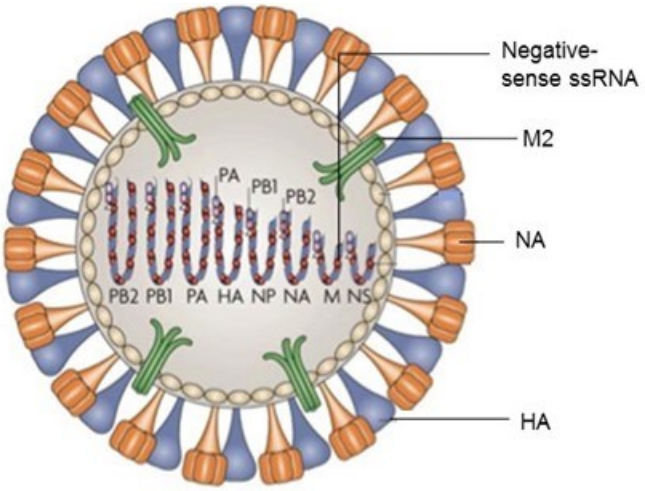
has remained a public health priority for over 50 years. Such efforts, however, hinge on a detailed understanding of virus biology, the host response to infection/vaccination and mechanisms adapted by the virus to evade host defenses.

The focus of the work presented in this dissertation is to advance our understanding of the host immune response to influenza vaccination and infection using human samples and mouse models of disease. The goal of the first chapter of this dissertation is provide the reader with an understanding of the current thinking regarding the immune mechanisms that drive protective and pathologic responses to influenza viruses. I will begin by providing an overview of influenza virus biology, antigenic diversity and clinical characteristics. I will then describe the current understanding of protective immunity to influenza and its utility in guiding vaccine design. This will be covered partly in Sections 1.2, 1.3 and 1.4. The second theme will focus on pathologic responses that lie at the heart of influenza pathogenesis and how understanding regulatory mechanisms that limit such responses can be beneficial in identifying new targets for intervention. This will be covered in Section 1.2.3

1.1.1 Virus biology and antigenic diversity

Influenza viruses are negative strand RNA viruses of the *Orthomyxoviridae* family and can be divided into three viral types: A, B and C (6). Of these, influenza A viruses exhibit the greatest genetic diversity and account for the vast majority of severe disease in humans and will therefore be the focus of this chapter. Influenza A viruses contain 8 single stranded, negative-sense, RNA gene segments that encode at least 11 proteins as shown in **Fig 1.1** (adapted from (7)).

Figure 1.1 The structure of influenza A virus



Adapted from Nelson M.I. and Holmes E.C.2007. Nat Rev Gen. 8:196-205

Fig 1.1 The genome of influenza A virus is composed of 8 gene segments and are listed from largest to smallest by convention. The first three gene segments (PB2, PB1 and PA) encode proteins that form the viral polymerase. The gene segment encoding PB1 also encodes a small mitochondrial protein PB1-F2 that is translated in another reading frame. Two segments encode the major surface envelope glycoprotein: Hemagglutinin (HA) which is responsible for receptor binding and entry into host cells and Neuraminidase (NA) which is responsible for budding new virions from infected cells. A single segment encodes nucleoprotein (NP) which binds the viral RNA. The seventh segment encodes two proteins: the matrix protein (M1) which serves as a major viral capsid protein and M2, a membrane protein that serves as an ion channel. The last segment also encodes two non-structural proteins: NS1 and NS2.

Influenza A viruses are further divided into subtypes based on the genetic sequence and serology of the two major surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There are currently 17 known HA (H1-H17) and 10 known NA (N1-

N10) subtypes (8). Viruses bearing all known HA and NA subtypes have been isolated from their natural reservoir, wild waterfowl. Avian influenza preferentially replicates in cells lining the intestinal tract of aquatic birds causing no symptoms or disease. The virus is then excreted in high concentrations in the feces and is thereby easily transmitted to other aquatic birds through the water supply (9). The avirulent nature of avian influenza infections in their natural hosts (wildfowl and shore birds) is likely due to centuries of virus adaption to this host. Interestingly, phylogenetic analysis of amino acid changes show that avian influenza viruses have low evolutionary rates with no evidence of net evolution over the last 60 years, suggesting that the virus may be in evolutionary stasis in this host (10). Occasionally, these avian subtypes can transmit to other species causing isolated outbreaks or can establish themselves in the new host. So far, avian influenza viruses have been shown to cause outbreaks in domestic poultry (such as chicken) and mammals including seals, whales, pigs and humans (reviewed in (11)).

In humans, the process by which new influenza subtypes carrying a novel hemagglutinin (or a novel hemagglutinin and neuraminidase) that is immunologically distinct from those of circulating influenza viruses, appear in the human population is called antigenic shift. Antigenic shift is thought to occur through the genetic reassortment of gene segments between human and avian influenza subtypes (12). Transmission from aquatic birds to humans was initially thought to require an intermediate animal host, such as pig, which can be infected by both human and avian species. However, recent infections of humans with the highly pathogenic H5N1 subtype as well as H7N9 suggest that domestic poultry, such as chicken and duck, can also serve as an intermediate host (13,14). Should the newly transmitted virus acquire the ability to be efficiently transmitted from person-person, a pandemic ensues. Due to the sheer number of influenza subtypes present in avian species as well as the large number of animal and avian reservoirs,

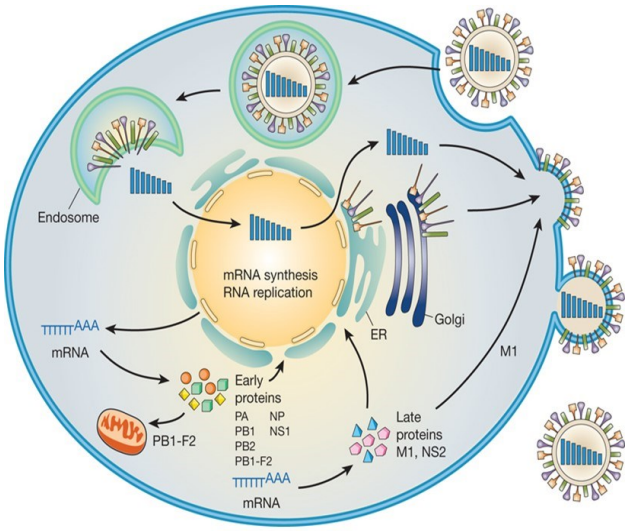
developing vaccines and other reagents against all influenza strains is not a feasible option (12). Thus increased surveillance of domestic birds and animals to detect new influenza subtypes as well as developing reagents, particularly vaccines that are effective across multiple different strains will be crucial in pandemic preparedness efforts. The latter will be discussed in more detail in coming sections.

Once established in the mammalian host, unlike avian viruses, mammalian influenza strains constantly undergo mutations by a process called antigenic drift. This occurs in both influenza A and B viruses (reviewed in (15)). Antigenic drift is a process by which the virus gradually accumulates point mutations including its two major surface proteins HA and NA. The mutations can be substitutions, deletions or insertions and occur throughout the viral genome resulting in the generation of antigenically divergent strains. Antigenic drift occurs because the viral RNA polymerase that transcribes the viral genome lacks proof reading activity thereby allowing non-deleterious point mutations to occur at a rate of roughly $1/10^4$ bases per replication cycle (16). The host humoral response selects for mutants with changes to HA and NA as these strains are able to avoid the neutralizing antibody response established by previous infection or vaccination, thereby allowing the generation of new seasonal variants capable of causing repeated infections in humans.

In humans, unlike avian species, influenza is primarily a respiratory disease (17). Person-to-person transmission occurs through aerosolized droplets generated by coughing, sneezing or talking. After inhalation, the virus gets deposited onto the respiratory tract, where it primarily infects the columnar epithelial cells of the upper respiratory tract. Influenza virus infection is mediated by binding of the receptor binding domain of the surface HA protein to the sialic acid (SA) present on glycans on the surface of host cells (18). The preference of HA subtypes to bind specific SA moieties is

important in determining host range and tissue tropism. Human adapted influenza viruses preferentially bind terminal SA linked to galactose by an α2,6 linkage, which are abundant in the respiratory epithelium of human airways (19-21). Avian influenza viruses, on the other hand, preferentially bind SA linked to galactose by an α2, 3 linkage, present on the respiratory and intestinal tracts of aquatic birds. After attachment, the virus enters the cell by endocytosis as shown in **Fig 2** (22). In the low pH of the endosome, the virus undergoes a conformational change thereby facilitating the fusion of the viral and endosomal lipid membranes releasing the viral ribonucleoproteins (RNPs) into the host cell cytoplasm (23). Once in the cell, viral RNPs are transported to the nucleus for vRNA replication and mRNA transcription using cellular machinery. Viral mRNAs are transported to the cytoplasm for translation into structural and non-structural proteins whereas vRNAs are translocated to the cytoplasm for virion assembly (24-26). Once assembled at the surface of the host cell, NA is required for release of the budding virion by cleaving the SA receptors facilitating the release of new virions (27).

Figure 1.2 Schematic diagram of influenza virus life cycle



From Neumann, G., et al.2009. Nature. 459: 931-939

Fig 1.2. After receptor-mediated endocytosis, the low pH of the endosome releases the viral RNA into the cytoplasm. Viral RNA is then transported to the nucleus wherein viral replication and transcription is carried out by the polymerase complex (PB1, PB2, and PA) and the nucleoprotein (NP). Messenger RNAs are transported to the cytoplasm for translation and early proteins that are required for transcription and viral replication are transported back to the nucleus.PB1-F2 associates with the mitochondria and is thought to induce apoptosis. Later in the viral life cycle, late proteins such as NS2 (also known as nuclear export protein or NEP) and the matrix protein M1 transport newly synthesized viral ribonucleoproteins from the nucleus. Virion assembly occurs at the host cell membrane and budding is facilitated by the neuraminidase activity of NA.

1.1.2. Clinical features of uncomplicated and complicated influenza

Seasonal influenza infections can vary in severity from subclinical infections to severe febrile illness (28). In adults and adolescents, influenza typically presents as a sudden onset of high-grade fever (38-40°C) with symptoms including chills, myalgia, dry cough, sore throat and headache. Bronchial airway hyper-reactivity and small-airway dysfunction are also common features. The fever peaks within the first 24 hours of infection and can last between 1-5 days during which time infectious virus particles are actively shed. These clinical features of uncomplicated influenza are generally indistinguishable from those of other respiratory viral infections. In children, gastrointestinal symptoms including nausea, vomiting, abdominal pain and diarrhea are also common (29) (30). While most healthy adults experience an acute infection that typically does not require medical intervention, chronic underlying medical conditions such heart and lung disease, diabetes and stroke can increase the risk of developing complications (31). Every year complications due to influenza infections are estimated to cause 36,000 deaths and more than 200,000 hospitalizations in the United States (2,32). The most frequent serious complications of influenza occur in the lung and fall into three main categories: primary viral pneumonia, secondary bacterial pneumonia and exacerbation of chronic pulmonary diseases.

Primary influenza pneumonia was first identified during the 1958-1959 pandemic (33) and is associated with a high mortality rate (6-20% during interpandemic periods) (34,35). Viral pneumonia can begin as early as 24 hours after the onset of febrile illness and is characterized by tachypnea, diffuse rales, cyanosis and ultimately respiratory failure (6,31). Unfortunately, patients often deteriorate despite treatment with antivirals. Histopathological findings in pure viral pneumonia include necrotizing bronchitis, intra-alveolar hemorrhage, edema and interstitial inflammation (36).

Secondary bacterial pneumonia was first identified during the 1918 pandemic and is characterized by the re-appearance of fever, dyspnea and productive cough during the convalescent phase of infection (37,38). The most common pathogens associated with secondary bacterial pneumonia are *Staphylococcus aureus*, *Haemophilus influenzae* and *Streptococcus pneumonia* (39). Like primary viral pneumonia, secondary bacterial pneumonia can also be associated with a high mortality rate of about 33% and has been observed during subsequent pandemics and seasonal epidemics.

In addition to pneumonia, influenza infections exacerbate pre-existing chronic pulmonary conditions such as chronic obstructive pulmonary disorder (COPD) and cystic fibrosis leading to excess morbidity and mortality in these patients (40). The exact mechanisms that underlie this process are not well understood (41).

1.2 The immune response to influenza

The severe lung disease caused by influenza, described in the previous section, poses a great challenge to the immune system: the host has to mount an effective immune response to rapidly eliminate the virus but tissue inflammation must be controlled to prevent immunopathology and respiratory failure. In this section, I will

review our current understanding of the role of innate and adaptive immunity in mediating protection from influenza. This summary will also serve as a primer for the next section on influenza vaccines and vaccine evaluation. Finally, I will discuss the role of the host immune response in the pathogenesis of severe influenza and highlight the importance of immune regulation in preventing the development of pulmonary injury.

1.2.1 The innate immune response

The cellular network of the lower respiratory tract (including the trachea, bronchi and lungs) is made up of CD45+ hematopoietic cells as well as CD45- stromal cells including various types of respiratory epithelial cells and fibroblasts. Of these, alveolar epithelial cells and epithelial cells lining the conducting airways serve as the primary target for influenza virus infection (42,43). Upon infection of permissive cells, the virus is first detected by cellular sensors namely, Toll-like receptors (TLRs), retinoic inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs), specifically NLRP3 (44,45). Engagement of these receptors on epithelial cells and resident innate immune cells including NK cells, lung resident dendritic cells (DCs) and alveolar macrophages activates them and triggers the release of pro-inflammatory cytokines including Type-1 interferons, IL-1β, IL-18, IL-6, TNF-α, IL-8 and chemokines such as CCL2 (reviewed in (46)). In humans, presentation of symptoms associated with influenza virus infection typically coincides with the onset and magnitude of the host innate immune response (47). IL-6, TNF-α and type 1 interferons (IFN-α) can be detected in symptomatic individuals both from nasopharyngeal washes as well as in serum and have been found to correlate with symptom score and body temperature (48).

The release of soluble mediators during the early phase of infection serves to enhance the antiviral capacity of infected cells and promotes the recruitment of inflammatory cells from blood and even distal compartments such as the bone marrow (49-51). Indeed, recruitment of NK cells (52), macrophages and neutrophils (53) have been shown to be critical in the control of viral replication. In addition to mediating early viral control, the innate immune response also plays a key role in regulating the development of adaptive immunity. Several pulmonary antigen-presenting cell (APC) populations including pDCs, monocyte derived DCs, alveolar macrophages and most notably resident alveolar and interstitial DC populations are thought to be important in the presentation of antigen to T cells in the draining lymph nodes (reviewed in (46) and (54)). In the case of lung resident DC's, virus uptake, either through phagocytosis of dying epithelial cells (55) or direct infection (56), triggers their mobilization and migration out of the lung. This process is also aided by cytokines such as type 1 interferons, IL-12 and TNF-α, which enhance DC maturation and migration (54,57). Activated migratory DCs upregulate the expression of costimulatory and adhesion molecules like CD80, CD86, CD40 and ICAM-1 as well as antigen presenting molecules making them potent APCs for the activation of the adaptive immune response.

1.2.2. The adaptive immune response

Cellular immunity

The migration of antigen-bearing mature APC's to the lymph nodes serves to activate naïve (and memory) T cells. Following their encounter with mature APC's, antigen-specific T cells undergo activation and proliferation and traffic to the lung as highly differentiated effectors, reaching peak numbers by 7-8 days post infection (58). In

the lung, activated CD4 and CD8 T cells mediate direct clearance of infected cells through multiple synergizing mechanisms including secretion of effector cytokines such as IFN-γ and TNF-α, exocytosis of cytolytic molecules such as perforin and granzyme and the expression of cell-death ligands such as Fas-L and TRAIL (59-61). In addition, CD4 T cell help is also thought to be critical for the generation of long-lived memory B cell and antibody responses required for effective and rapid clearance during re-infection (62-64). Following clearance of virus, there is a rapid contraction of antigen-specific T cells leaving only a small number of memory cells that can persist long term (65). The memory T cell response to re-infection has been shown to have a profound effect on protection in two primary ways:

1. T cell mediated “help” for antibody production

Recently, a dedicated subset of T follicular helper (T_{FH}) cells that are required for the formation of germinal centers and the generation of long-lived serological memory were described (reviewed in (66) and (67)). Germinal center T_{FH} cells have been shown to be required for the maintenance and proliferation of germinal center B cells and for the differentiation of germinal center B cells into plasma and memory B cells. In animal studies and recently in humans, induction of T_{FH} cells by vaccination has been found to correlate with increased antibody production (68,69).

The generation of long-lived B cell memory is critical to the prevention of re-infection and is the goal of most current vaccination strategies and will be described in more detail in the following section. As illustrated above, the induction of T helper cells responses is increasingly recognized as being critical to this process and could be an important part of new vaccine efforts, particularly in vulnerable populations such as the elderly where poor antibody responses to vaccination and infection have been observed (70).

2. Direct control of viral replication

In addition to providing “help” for antibody production, T cell memory has been shown to play an important role in mediating direct control of viral load. Antigen specific memory CD4 and CD8 T cells are recruited to the site of infection within 2-3 days following secondary challenge and can rapidly produce effector cytokines upon cognate antigen recognition (71,72). The early production of cytokines and chemokines serves to mobilize and activate innate cells, which can further contribute to control of viral replication (73,74).

Epitope mapping efforts suggest that unlike antibodies, which primarily recognize epitopes from the highly variable surface proteins (HA and NA), T cells recognize epitopes derived from the internal proteins of the virus that are more conserved between divergent influenza strains (75). Indeed, in animal models of infection, T cells have been shown to be the primary mediators of cross-protective or heterosubtypic immunity (76). Despite studies demonstrating the importance of memory T cells in protective immunity, particularly cross-reactive immunity to influenza in animal models, until recently, relatively little was known about their importance in human disease. Influenza-specific T cells can be readily detected in peripheral blood in healthy adults, however, evidence for a role in protective immunity is sparse and largely circumstantial. In early studies of experimentally infected humans, cytotoxic T cells correlated with viral clearance in individuals lacking antibodies, although these studies did not distinguish CD4 and CD8 T cells (77). More recently, in a controlled challenge study, pre-existing memory CD4 T cells were found, for the first time, to be associated with protection from disease in the absence of neutralizing antibodies (78). The magnitude of the CD4 T cell response to infection correlated with reduced viral shedding, reduced disease severity and duration.

Consistent with findings in animal models (79), influenza specific CD4 T cells were able to exert direct cytolytic effects on infected cells. In humans, numerous studies have demonstrated the existence of pre-existing memory CD4 and CD8 T cells that recognize influenza subtypes to which individuals have had no prior exposure (75,80). A recent study with over 300 participants showed that the frequency of pre-existing cross-reactive memory CD8 T cells correlated inversely with symptom score during the 2009 pandemic (81). This landmark study is the first to demonstrate the importance of these cross-reactive T cells in mediating heterotypic protection against newly emergent strains in humans. These studies strengthen the argument that harnessing cross-reactive cellular immunity maybe of great benefit in designing vaccines to combat newly emergent influenza subtypes.

Humoral immunity

Like T cells, B cells responses are also primarily induced in the draining lymph nodes either through interaction with antigen-bearing DCs or through direct uptake of antigen (82-84). In mice, antibody forming cells can be detected as early as 3 days after infection in the draining lymph nodes and are soon followed by the presence of neutralizing antibodies in nasal and lung lavages. Neutralizing antibodies directed against the globular head of the surface hemagglutinin (HA) are considered the most effective mediators of protection against influenza and are the widely used as the primary immune correlate of protection from re-infection in humans (85). However, due to the highly variable nature of the globular head of HA, anti-HA antibodies have been shown to be largely strain-specific. Interestingly, several recent studies have identified antibodies that recognize epitopes derived from the more conserved stalk region of HA as well as epitopes from other conserved proteins including M2 (86-88). While the relative

contribution of these cross-reactive antibodies to protective immunity during natural infection in humans is still unclear, there is interest in developing vaccines that can selectively induce antibodies to such well conserved epitopes and is discussed further in section 1.3.5.

1.2.3 Regulating the immune response to influenza

The development of an effective immune response (as described above) is critical to viral control and prevention of virus mediated lung injury. However, appropriate regulation of the immune response is as important in preventing host-mediated immunopathology. In this section, I will provide a review of data that implicate the immune response as central to influenza pathogenesis as well as highlight the current understanding of regulatory mechanisms that govern this process.

Despite their critical role in controlling viral replication, studies in animals as well as humans suggest that the host immune response maybe instrumental in causing pulmonary pathology during seasonal and pandemic influenza infections. Experimental studies in mouse and non-human primate models demonstrate a profound and early over-expression of cytokines and chemokine after infection with highly virulent strains of influenza (89). Similarly, individuals who succumbed to disease either during infection with the highly pathogenic H5N1 virus or during severe seasonal infections had high levels of proinflammatory mediators including IL-6, CCL2, CXCL10 (IP-10) and IL-8 (90) (91) (92). This phenomenon is referred to as the “cytokine storm” and has been observed with other acute respiratory viruses including those in the coronavirus family (severe acute respiratory syndrome virus - SARS and Middle East Respiratory Syndrome coronavirus – MERS-CoV) (93,94). Uncontrolled production of chemokines,

particularly CCL2, has been found to induce increased recruitment of innate immune cells such as monocytes/macrophage and NK cells into the lung, which in turn release more cytokines, thereby exacerbating the cytokine storm (95,96). The increased recruitment of TNF-α and iNOS producing dendritic cells in particular has been shown to be associated with severe influenza in animal models (97). This exacerbated proinflammatory response is believed to contribute to severity of the disease by leading to host-mediated collateral damage of host tissue, acute respiratory distress syndrome (ARDS) and multiple organ failure (98,99).

Undoubtedly, stringent regulation of the above-described inflammatory response is critical to minimizing immunopathology and both innate and adaptive immune cells have been found to contribute to this process. As mentioned previously, neutrophils are thought to be important contributors to tissue pathology during influenza. However, in mice, neutrophil depletion during influenza led to exacerbated pulmonary inflammation, edema and hemorrhage suggesting that neutrophils may also play a regulatory role during infection in vivo, although the exact mechanisms remain unclear (53,100). Similarly, lung epithelial cells have been shown to express the macrophage inhibitory molecule CD200 to dampen down pulmonary inflammation (101). Classically activated macrophages are thought to play a role in pulmonary injury by secreting high levels of inflammatory mediators (102). However, two other populations of macrophages have been described in the literature, namely wound healing macrophages and regulatory macrophages, together referred as alternatively activated macrophages (103). Activation of the nuclear receptor peroxisome proliferator activated receptor (PPAR)-γ, an important regulator of the alternatively activated phenotype (104), seems to alleviate pulmonary inflammation during lethal influenza infection, suggesting that alternatively activated macrophages may play a role in resolution of inflammation (97,105). However,

more studies are required to better understand if and how alternatively activated macrophages control tissue damage during influenza.

The anti-inflammatory cytokine, IL-10, has long been recognized as one of the most potent negative regulators of the immune system. It is expressed by a wide variety of cell types including macrophages, neutrophils, epithelial cells, B cells and importantly T cells (reviewed in (106)). IL-10 is thought to act primarily through the suppression of APC function and activation, thereby limiting proinflammatory cytokine secretion and induction of $T_{effector}$ cells. Although IL-10 is critical in controlling inflammation and tissue damage during several infectious as well as autoimmune diseases, surprisingly little is known about its role during influenza infections in humans or animal models. Somewhat unexpectedly, several recent studies show that expression of IL-10 is associated with severe disease and poor outcomes during influenza infections in humans (107-109). In animal models, the role of IL-10 is less clear with one study demonstrating a protective role for IL-10 (110) while others have reported a pathogenic role (111,112). Resolving these conflicting data will be important to better understanding regulatory mechanisms that govern the development of protective pulmonary responses versus immunopathology. This will be the focus of the 4th chapter of this dissertation.

As mentioned previously, infection with influenza results in the loss of respiratory epithelial cells resulting in significant respiratory dysfunction (reviewed in (113)). Exciting new research suggests that a rare population of immune cells termed innate lymphoid cells (ILCs) may play a critical role in the repair of this tissue injury (114). ILCs can be broadly divided into 3 functional categories: NK cells, RORγt^{+} ILCs and type 2 ILCs (ILC2). IL-22 expressing NK cells (115) as well as ILC2 that make T helper 2 (T_H2) cytokines including IL-4, IL-5 and IL-13 (116) have been found to be important in regulating epithelial integrity and promoting tissue homeostasis after infection with

influenza. Importantly, similar observations have been made in other mucosal tissues (117,118) as well as in non-infectious models of injury (119). These data suggest that this may represent an evolutionarily conserved mechanism to maintain and promote barrier integrity at mucosal sites and warrants further study.

Targeting host inflammatory and/or repair pathways represents an exciting opportunity to limit tissue injury associated with infectious or chronic inflammatory conditions. We are just beginning to appreciate that immune cell signaling not only controls the initiation of inflammation but may also play a critical role in the initiation of tissue repair and remodeling. Understanding these divergent yet related pathways is critical to the identification of new targets for therapeutic manipulation.

1.3 Vaccines

Vaccines are currently the most effective means to control the burden of influenza (6). While originally thought to confer protection to between 60-90% of vaccine recipients, emerging data suggest that vaccine efficacy maybe lower than previously thought, particularly in vulnerable populations. This section is intended to provide a review of the current strategies for commercial seasonal influenza vaccine manufacture, vaccine efficacy and effectiveness as well as new strategies that are under investigation to improve vaccine efficacy. Finally, I will discuss vaccine evaluation strategies as an important aspect of new vaccine development.

The current seasonal influenza vaccines are designed to induce strain-specific neutralizing antibodies against the variable HA protein (85). To account for the constant drift within HA, the seasonal vaccines are reformulated ever year based on year-round global surveillance of influenza strains in the population, accomplished through the WHO

Global Influenza Surveillance and Response System (120). Once the dominant circulating strains have been identified, WHO collaborating centers generate a vaccine seed strain through genetic re-assortment of a master strain and the field strains, which are then distributed to the vaccine producers.

In the United States, there are currently three types of influenza vaccine formulations that are available commercially: inactivated influenza vaccines, live attenuated influenza vaccines and a recombinant influenza vaccine (which will become available for distribution during the 2013-2014 influenza season)(121). Commercially available influenza vaccines in the United States are listed in Table 1 (adapted from (121)).

Table 1.1 List of seasonal influenza vaccines-United States, 2013-14 season (121)

Vaccine	Trade name	Manufacturer	Age indications	Route
IIV3†††, Standard Dose	Afluria®	CSL Limited	≥9 yrs.	IM
	Fluarix®	GlaxoSmithKline	≥3 yrs.	IM
	Flucelvax®§§§	Novartis Vaccines	≥18 yrs.	IM
	FluLaval®	ID Biomedical Corporation of Quebec (distributed by GlaxoSmithKline)	≥18 yrs	IM
	Fluvirin®	Novartis Vaccines	≥4 yrs.	IM
	Fluzone®	Sanofi Pasteur	6 through 35 mo.	IM
			≥36 mo.	IM
			≥36 mo.	IM
			≥6 mo.	IM
	Fluzone® Intradermal§	Sanofi Pasteur	18 through 64 yrs.	ID
IIV3†††, High Dose	Fluzone® High-Dose**	Sanofi Pasteur	≥65 yrs.	IM
IIV4†††, Standard Dose	Fluarix® Quadrivalent	GlaxoSmithKline	≥3 yrs.	IM
RIV3†††	FluBlok®	Protein Sciences	18 through 49 yrs.	IM
LAIV4†††	FluMist® Quadrivalent††	MedImmune	2 through 49 yrs.	IN

Abbreviations: IIV=Inactivated Influenza Vaccine; IIV3=Inactivated Influenza Vaccine, Trivalent; IIV4=Inactivated Influenza Vaccine, Quadrivalent; RIV3=Recombinant Influenza Vaccine, Trivalent; LAIV4=Live, Attenuated Influenza Vaccine, Quadrivalent; IM=intramuscular; ID=intradermal; IN=intranasal.

§ Trivalent inactivated vaccine, intradermal: A 0.1-mL dose contains 9 mcg of each vaccine antigen (27 mcg total).

** Trivalent inactivated vaccine, high-dose: A 0.5-mL dose contains 60 mcg of each vaccine antigen (180 mcg total).

†† It is anticipated that the quadrivalent formulation of FluMist® will replace the trivalent formulation for the 2013-2014 season.

††† Trivalent influenza vaccines contain three different vaccine viral antigens, one each from an influenza A(H1N1) virus, an influenza A(H3N2) virus, and an influenza B virus. Quadrivalent influenza vaccines contain the same three antigens as trivalent vaccines, along with an antigen from a second influenza B vaccine virus strain.

§§§ Cell culture-based trivalent inactivated influenza vaccine (ccIIV3)

1.3.1 Inactivated influenza vaccines

The inactivated influenza vaccine first became approved for use in the United States in 1945 and is still widely used today (122). The vaccines were originally produced by propagation of influenza viruses in embryonated hens' eggs followed by inactivation and purification. Today, inactivated influenza vaccine seed strains are obtained by re-assorting the HA and NA of field strains with internal gene segments from a "high growth" master donor strain (A/Puerto Rico/8/34) (123).There is no "high growth" influenza B master donor strain currently available. While the majority of inactivated vaccines are still produced by propagating the vaccine seed strain in hens' eggs, depending on the extent of purification, the inactivated vaccines can be divided into 3 types: whole virus, split and subunit vaccines. In the United States, none of the commercially available vaccines contain adjuvants.

1. Whole virus vaccines

Whole-virus inactivated, unadjuvanted vaccines are manufactured by inactivating purified vaccine seed strains with formaldehyde or β propiolactone (BPL) followed by purification. While whole-virus vaccines are reported to be more immunogenic in naïve populations and are still used in some countries, they have been associated with a higher frequency of adverse reactions compared with other influenza vaccines, particularly in children (123). Therefore, they are estimated to account for less than a third of all influenza vaccine production and are not manufactured in the United States.

2. Split vaccines

The majority of inactivated influenza vaccines are “split” by treating the purified vaccine seed strain with a detergent such as Triton X-100, sodium lauryl sulphate or Tween 80 to extract proteins from the lipid membranes. The preparation is then further purified and the HA rich fragment is harvested. The split vaccines are administered intramuscularly with the most commonly reported adverse events being injection site reaction followed by pain, fever, myalgia and headache (124). In 2011, the US FDA approved Sanofi Pasteur’s Fluzone® intradermal vaccine (125).

3. Subunit vaccines

The preparation of subunit vaccines is very similar to that of split vaccines, but more rigorous purification is carried out so that the vaccine consists almost exclusively of the purified HA and NA subunits with minimal contaminating N, matrix protein, nucleoprotein and lipid. All subunit vaccines are currently administered intramuscularly.

1.3.2 Live attenuated influenza vaccines (LAIV)

The first intranasally delivered, live attenuated influenza vaccine (FluMist®) was licensed in the United States in 2003 and was developed to more closely resemble natural infection compared to the parenterally delivered inactivated vaccines (126). The live attenuated seed vaccine strains are constructed by re-assorting the HA and NA gene segments from circulating influenza strains with 6 gene segments from a master donor strain which is temperature sensitive (ts), cold-adapted (ca) and attenuated (att). In the United States, two master donor strains are used in the commercial manufacture

of FluMist® : A/Ann Arbor/6/60 and B/Ann Arbor/1/66. The most common side effect after administration of LAIV is runny nose and nasal congestion (127,128).

1.3.3 Recombinant influenza vaccines

A new recombinant trivalent influenza vaccine (Flublok®) was approved in 2013 and will be available for distribution during the 2013-2014 influenza season. Unlike existing influenza vaccines that involve growing influenza viruses in hens' eggs or mammalian cell lines, requiring longer production time and are costly, the recombinant vaccine is manufactured by expressing each of the three selected HAs in a continuous insect cell line using a baculovirus expression system. The HAs are then extracted using Triton X-100 and purified by column chromatography before packaging (http://www.fda.gov/downloads/BiologicsBloodVaccines/Vaccines/ApprovedProducts/UCM336020.pdf).

1.3.4 Efficacy and effectiveness of seasonal influenza vaccines

Early estimates of seasonal influenza vaccine efficacy suggested that the seasonal influenza vaccines were between 70-90% effective in preventing seasonal influenza-like illness (123,129-132). However, the true effectiveness of seasonal vaccines has recently been the topic of intense debate with a recent meta-analysis revealing much lower vaccine efficacy than previously thought ((133)). In this section, I will outline the most current information regarding the efficacy of the seasonal trivalent inactivate vaccine (TIV) and the live attenuated influenza vaccine (LAIV) in different age groups.

Children

In the United States, inactivated influenza vaccines are recommended for all children ≥6 months of age whereas LAIV is only approved for use in children older than 2 (134). Studies with age-specific data suggest limited effectiveness of the inactivated vaccine against medically attended, laboratory-confirmed influenza in children with the lowest vaccine effectiveness (7%-52%) noted in children aged 6-59 months (123,135-138). In years of suboptimal antigenic match between vaccine and circulating strains, the inactivated vaccine was not found to be significantly effective against influenza related illness.

On the other hand, LAIV has been found to be highly efficacious in young children, providing significant protection (pooled vaccine efficacy of 83%) against laboratory-confirmed influenza in years with good antigenic match but importantly even in years when there was suboptimal match between circulating and vaccine strains (133).

Adults <65

In healthy, young adults, seasonal TIV and LAIV were previously shown to prevent laboratory confirmed influenza in 70%-90% of healthy adults under 65 years of age (130,139). However, a recent meta-analysis of ten randomized controlled trials over twelve influenza seasons suggests that the pooled vaccine efficacy of TIV in preventing laboratory-confirmed influenza was only 59% (133). This analysis is supported by more recent data from observational studies suggesting lower effectiveness of TIV in preventing influenza infections in healthy adults (140). Additionally, efficacy and effectiveness in preventing illness have been shown to be substantially lower in studies conducted during influenza seasons when the vaccine and circulating strains were not antigenically matched (131). Surprisingly, no protective efficacy was observed after LAIV in any of the randomized controlled trials (133).

Vaccine effectiveness among adults with chronic diseases has also been shown to be lower than that for healthy adults (123).

Adults >65

Adults>65 represent an important population who account for more than 90% of the mortality associated with seasonal epidemics every year (141). Currently only the inactivated vaccine is approved for use in this population. A recent review of the efficacy of effectiveness of seasonal vaccination suggests that in elderly individuals living in the community, vaccines were not significantly effective against influenza, influenza-like illness or pneumonia (142) and were only moderately effective (vaccine effectiveness 23%) against influenza-like illness in elderly living in nursing homes and long-term care facilities. Consistent with poor effectiveness in preventing illness, the current vaccines have also been shown to induce weak antibody responses in the elderly (134). In an effort to increase immunogenicity and vaccine effectiveness, a high dose formulation has recently been approved for use in individuals over 65 (143). The higher dose preparations have been shown to be more immunogenic and elicit a significantly higher antibody response although their effectiveness in preventing infection is yet to be determined. An MF59 adjuvanted vaccine (FLUAD^{TM} Novartis) has also been developed primarily for use in the elderly. Interestingly, early clinical studies indicates significantly enhanced protection against influenza in this population (144).

Taken together, these data suggest while the current vaccines are efficacious in preventing infection under certain circumstances, over all there is a need for more effective vaccines. In the next section, I will outline some of the advances in the development of next-generation influenza vaccines.

1.3.5 Advances in seasonal and pandemic influenza vaccines

As detailed above, even when influenza vaccines are well matched to circulating viruses, their effectiveness in preventing illness in individuals who are at the greatest risk of severe disease, namely the elderly, young children and individuals with underlying medical conditions is thought to be low (133,134,136). Even in healthy adults, vaccine efficacy in preventing febrile illness has recently been shown to be lower than what was previously shown. In addition, the current vaccines are thought to be poorly efficacious against newly emergent or drifted influenza strains making yearly reformulation of seasonal vaccines a necessity. In the event of an influenza pandemic, as was observed in 2009, there can a significant delay between the emergence of a pandemic strain and the development of an effective pandemic vaccine, partly due to production time required for current egg-based technologies. For these reasons, there is considerable interest in the development of more immunogenic and broadly cross-reactive vaccines for use in all age groups. Recently, a self-assembling influenza nanoparticle vaccine that can offer broadly cross-reactive immunity has been described (145). An advantage of the virus-like particle (VLP) approach over existing platforms is that manufacture of VLP vaccines does not require live influenza viruses for development or manufacture. Additionally, they are predominantly produced using a baculovirus/insect cell-expression system that serves as high-yielding low-cost commercial alternative to egg-based or mammalian cell line-produced influenza vaccine approaches.

Adjuvants represent an important means to enhance the immunogenicity of vaccines. Use of adjuvants in vaccine formulations has been shown to allow dose sparing, promote epitope spreading and enhance immunogenicity of the vaccines (146,147). While, in the United States, the seasonal influenza vaccines are unadjuvanted, oil-in-water emulsion adjuvants (MF59) for pandemic and seasonal

influenza are currently used in over 20 counties around the world (148). The success of MF59 has promoted research into other oil-in-water formulations. In addition, TLR agonists are also being tested for safety and immunogenicity (149).

The development of the so-called “universal” influenza vaccine which can offer cross-reactive immunity against multiple influenza subtypes is the holy grail of influenza vaccines. There are broadly speaking two approaches that are actively being pursued to achieve this goal: antibody-based strategies and T-cell based strategies. Antibody-based strategies aim to induce antibodies against conserved regions of surface protein, including the stem region of HA (145,150) and extracellular domain of M2 (151). Pre-clinical studies in small animals show that antibodies against the conserved regions of surface proteins have the ability to prevent infection from a number of influenza subtypes (152). However, a recent study demonstrated that antibodies binding to conserved regions of HA may enhance fusion with host cell membrane, thereby highlighting a potential limitation of such an approach (153).

The second strategy is based on recent findings in humans that point to an important role for T cells in mediating protective and most notably cross-reactive immunity to influenza (78,81). However, our limited understanding of the induction and maintenance of T cell memory, particularly in humans will be a major challenge in the design of such T cell inducing vaccines. To this end, the goal of chapter 3 of this thesis was to characterize influenza-specific T cell responses in humans as well as examine the impact of different vaccination strategies on T cell immunity.

A major component of new vaccine development efforts is identifying quantitative measures of immunological responses that correlate with protection (154). In the next section, I will describe the current paradigm that governs clinical vaccine evaluation as well future prospects for new influenza vaccine testing.

1.3.6 Vaccine evaluation and correlates of protection

Currently, anti-HA antibodies are the only established immune correlate of protection and are the gold standard for measuring vaccine immunogenicity. Antibodies against influenza are routinely measured in the serum by the hemagglutination inhibition assay (HAI). Numerous studies have shown that on a population level, pre-exposure HAI titers of $\geq$ 32 to 40 are associated with protection from re-infection and is often referred to as the seroprotective titer (155,156). In fact, the higher the HAI titer, the higher the rates of protection (157). Due to the strong correlation between HAI titers and protection from infection, the current vaccines are evaluated almost exclusively on the basis of their ability to induce a 4-fold increase in serum antibody titers against vaccine strains following vaccination compared to pre-vaccination titers. More recently, however, it has been recognized that such serological endpoints may not fully reflect vaccine immunogenicity or accurately predict protection from infection. In support of this, a recent study found high HAI titers in individuals who were not protected following vaccination with TIV or LAIV suggesting that in addition to quantity, perhaps the quality of the antibody response may dictate protection from infection (158). Alternatively, immune parameters besides antibodies may contribute to protective immunity (85). This maybe particularly relevant in the case of LAIV, wherein serum antibody titers do not correlate with protection from infection (134). Despite being as efficacious as TIV in preventing infection (127), several groups have noted poor serum antibody responses in LAIV recipients suggesting that mucosal antibodies and/or other immune mechanisms such as influenza-specific T cells may contribute to protection after immunization. In fact, till date, there are no correlates of protection following LAIV.

As mentioned previously, the development of a cross-reactive “universal” influenza vaccine is of great interest from a public health standpoint and yet, current influenza vaccines are evaluated solely based on their ability to induce strain-specific anti-HA antibodies. A more comprehensive evaluation of current and new vaccines in terms of their ability to induce cross-reactive responses may help predict their ability to prevent infection from newly emergent strains.

CHAPTER 2: Serum antibody and cytokine response to TIV and LAIV

2.1 Abstract

Despite vaccine efforts, influenza outbreaks pose a significant threat to global public health. There are two types of commercially available seasonal influenza vaccines in the United States: the trivalent inactivated vaccine (TIV), delivered parenterally, and the trivalent live attenuated influenza vaccine (LAIV), delivered intranasally. Although both vaccines are generally efficacious, the immunologic mechanisms which contribute to protective immunity are incompletely understood. Thus, we investigated the effects of TIV and LAIV on serum cytokine profiles in healthy adults at 14 and 28 days post-vaccination over two influenza seasons and examined their relationship to serum antibody induction. We found that TIV recipients had a significantly higher sero-response rate compared to LAIV recipients, as has previously been shown. Interestingly, vaccination with TIV was also associated with a small, yet significant, decrease in the levels of IL-8 and TNF-α at 14 and 28 days post-vaccination. However, LAIV had no impact on serum cytokine levels at these time points. Finally, we examined the relationship between baseline serum cytokine levels and antibody responses to TIV (due to the poor sero-response rate in LAIV recipients). In TIV recipients, pre-vaccination levels of IL-8 were positively correlated with sero-response to TIV. These data provide insight into the systemic immune response to different vaccine formulations and/or sites of delivery which may be useful to new vaccine strategies.

2.2 Introduction

Influenza outbreaks remain a major cause of morbidity and mortality worldwide. Seasonal influenza A infections result in 3-5 million cases of severe infection and between 250,000 and 500,000 deaths around the world annually (159). In the United States alone, approximately 200,000 people are hospitalized every year in seasonal outbreaks. Currently, vaccination is the most effective means to control the spread of seasonal influenza (160). Until recently, there were only two types of vaccines available in the United States: The live attenuated influenza vaccine (LAIV) which is currently approved for use in immune-competent children and adults ranging from 2 to 49 years and the trivalent inactivated vaccine (TIV), which is approved for use in most individuals including the very young and the elderly. Both vaccines have been shown to be safe and anywhere between 40-100% effective in young, healthy adults (134). However, vaccine efficacy can be influenced by age, underlying medical conditions as well as antigenic similarity between the circulating and vaccine strains

Since TIV and LAIV differ in both the route of administration (parenteral versus mucosal) as well as the formulation (killed versus live), the immune responses to these vaccines are likely to be different (160,161). For instance, intramuscular injection with TIV has been shown to induce a robust increase in serum antibodies, a widely used correlate of protection (162-164). In contrast, vaccination with LAIV induces a substantially lower serum antibody response despite conferring similar levels of protection from infection in adult vaccine recipients. These data suggest that multiple arms of the immune response including T cells, cytokines, and mucosal antibodies may contribute to protective immunity, particularly after vaccination with LAIV. However, the current vaccines are almost exclusively evaluated based on their ability to enhance serum antibody titers. Therefore, our understanding of the immune response to LAIV

and TIV is not complete. As new vaccines and delivery strategies are rapidly under development to combat seasonal and pandemic influenza as well as other infectious diseases, there is an urgent need to identify the immunologic responses which underpin efficacious vaccine strategies (15,165).

Cytokines are important immune mediators that serve to orchestrate the immune response to natural infection and vaccination (166). Analysis of cytokines in infected animals and humans have provided important clues into the immune mechanisms that mediate protection as well as recovery from influenza and other infectious agents (167-169). In contrast to their wide use in understanding the immune response in a pathological setting, changes in cytokine profiles after vaccination have not been well characterized. However, studies in both animals and humans have shown that vaccine-induced changes in serum cytokines correlate with T cell and innate cell activation (170-172). These data suggest that changes in global cytokine profiles after vaccination may provide useful insight into vaccine efficacy and could potentially serve as biomarkers of immune activation following vaccination (171-173). Accordingly, in this study we sought to understand how different seasonal influenza vaccine formulations/deliveries (i.e. TIV and LAIV) influence serum cytokine profiles in healthy adults. We also determined the relationship between serum cytokines profiles and the development of antibody responses following vaccination.

2.3 Materials and methods

Study design and human subjects

A convenience sample of adults between the ages of 18 and 49 were enrolled at the influenza vaccine clinic at the Johns Hopkins University Hospital from October 1 until November 30 of both the 2006-07 and 2007-08 influenza vaccine seasons. Participants were screened by questionnaire to assess health history including recent acute illness. Volunteers were excluded from the study if they reported to be immuno-compromised due to chronic illness or medication. This study was approved by the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health.

All volunteers provided written informed consent and chose their vaccine type: LAIV (Flumist, Medimmune) or TIV (Fluzone, Aventis Pasteur). Immediately prior to vaccination (day 0), a venous blood specimen of approximately 50 ml was collected. Volunteers were then immunized following current guidelines for influenza vaccination. Participants provided additional blood samples at 14 (day 14) and 28 days (day 28) after vaccination. Serum was harvested from blood samples using serum separator tubes (Becton Dickinson, San Jose, CA) and stored at -80˚C. The 2006-07 seasonal vaccines contained A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. In the 2007-08 season, both vaccines contained A/ Solomon Islands/3/2006 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004. Each dose of TIV contained 45 µg of HA in the recommended ratio of 15 µg from each virus strain. Each dose of LAIV contained $10^{6.5}$ to $10^{7.5}$ median tissue culture infectious doses ($TCID_{50}$) of live attenuated viruses of each of the three strains.

Hemagglutination inhibition assay (HI)

HI titers were measured in serum samples for all three vaccine strains in each season. Influenza virus strains A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 and A/ Solomon Islands/3/2006 (H1N1) were obtained from the CDC. Serum samples were pretreated with receptor destroying enzyme (Lonza Inc., Walkersville, MD) overnight at 37˚C. Samples were then treated with Sodium Citrate (1.6%) and heated at 56˚C for 45 minutes. The hemagglutination inhibition (HI) assay was performed as described previously (162). Briefly, 25 µl of specific influenza virus (4 HA units) was added to 25 µl of serum serially diluted in 2-fold dilutions till 1:2048 in PBS and incubated for 1 hour at room temperature. 50 µl of 0.5% chicken red blood cells (for influenza A H1N1 and influenza B) and turkey red blood cells (for influenza A H3N2) was then added and incubated for a minimum of 30 minutes before reading the HI activity. All erythrocytes were obtained from CBT farms (Federalsburg, MD). The HI antibody titer for a given sample was defined as the reciprocal of the last dilution at which there was complete inhibition of HA activity. For example, if the last dilution at which there was a complete inhibition of HA activity was 1:4, the titer for the given sample was represented as 4. A titer of 2 was assigned to all samples in which the first dilution (1:4) was negative.

Serum cytokine quantification

Serum cytokines were determined using the Ultrasensitive Human Th1/Th2 10-plex from Meso-Scale Discovery (MSD), (Gaithersburg, MD). The MSD multispot array was run according to the manufacturer's protocol with minor modifications. Briefly, plates were pre-incubated with 25 µl of supplied human sera diluents for 30 minutes, with shaking, at room temperature. A modified 12-point calibration curve was used instead of

the recommended 8-point curve (174). Calibration curves were prepared in the diluents and ranged from 2500 pg/ml to 0.15 pg/ml. Following the 30 minute incubation period, 25 µl of serum sample or calibrator was added to the wells in duplicates. Plates were then incubated at room temperature for 2 hours with shaking. Plates were then washed with PBS and 0.05% Tween-20 and then incubated with 25 µl of capture antibody for 2 hours at room temperature with shaking. After washing plates with PBS and 0.05% Tween, 150 µl of detection antibody was added. Plates were read using the MS2400 imager (MSD).

The lowest limit of quantification (LLOQ) was defined as the lowest calibrator value at which the coefficient of variance of concentration was less than 30% and recovery of calibrator was within 30% of the expected value (as suggested by the manufacturer). All cytokine values that were below the LLOQ were considered undetectable and assigned a value equal to the plate-specific LLOQ for statistical analyses.

Statistical Analyses

To determine differences in proportions and means, the Fischer's exact chi square and the t test statistic were used, respectively. The Wilcoxon rank sign test was used to compare median cytokine levels of TIV and LAIV users at day 0; the paired test was used to compare the median levels of each cytokine at day 0 to day 14 and day 28. A serologic response to vaccine was defined as a ≥4-fold increase in titers compared to day 0. A two-sided p-value of <0.05 guided statistical interpretation. All analyses were conducted in SAS 9.2 (Carey, North Carolina).

2.4 Results

2.4.1 Study population

A total of 42 and 58 volunteers were recruited and enrolled in the 2006-07 and 2007-08 influenza seasons, respectively. Nine individuals from the first year also participated in the second year of the study. Demographic characteristics of LAIV and TIV recipients were similar in the 2006-07 influenza season (**Table 2.1**). In the 2007-08 season, a higher proportion of men received LAIV than TIV (62% vs. 32%, p=0.05), and greater number of individuals who had never been previously vaccinated received LAIV compared to TIV (38% vs. 6%, p<0.01; **Table 2.1**). Due to a delay in the availability of LAIV in the 2007-08 season, LAIV recipients were recruited slightly later (November only) compared to the TIV recipients (October-November) during the 2006-07 season.

Table 2.1: Characteristics of study participants at baseline, by influenza season and vaccination type

	2006-07 season					2007-08 season				
	TIV (N=25)		LAIV (N=17)			TIV (N=31)		LAIV (N=21)		
Characteristic	n	%	n	%	p-values†	n	%	n	%	p-values†
Male	10	40%	7	41%	>0.99	10	32%	13	62%	**0.05**
Female	15	60%	10	59%	>0.99	21	68%	8	38%	**0.05**
Age, mean (SD)	29.5	(7.2)	29.5	(7.2)	>0.99	28.9	(7.1)	30.1	(7.0)	0.96
Fever	0	0%	0	0%	--	0	0%	0	0%	--
Rash	0	0%	0	0%	--	0	0%	1	5%	0.40
Runny Nose	2	8%	3	18%	0.38	3	10%	1	5%	0.64
Cough	2	8%	1	6%	>0.99	0	0%	0	0%	--
Diahrrea	1	4%	0	0%	>0.99	2	6%	0	0%	0.51
Vomiting	0	0%	0	0%	--	0	0%	0	0%	--
Other symptoms	2	8%	0	0%	0.51	2	6%	0	0%	0.51
On medication‡	8	32%	3	18%	0.48	9	29%	2	10%	0.17
On birth control	6	40%	2	20%	0.44	8	38%	1	13%	0.37
Never received a flu vaccination	--	--	--	--	--	2	6%	8	38%	<0.01
Sero-positive against A/H1N1	25	100%	17	100%	-	30	96%	20	95%	>0.99
Sero-positive against H3N2	10	80%	11	65%	**0.02**	29	94%	18	86%	0.09
Sero-positive against Flu B	23	90%	15	88%	0.82	31	100%	21	100%	-

‡Medications excluding birth control.

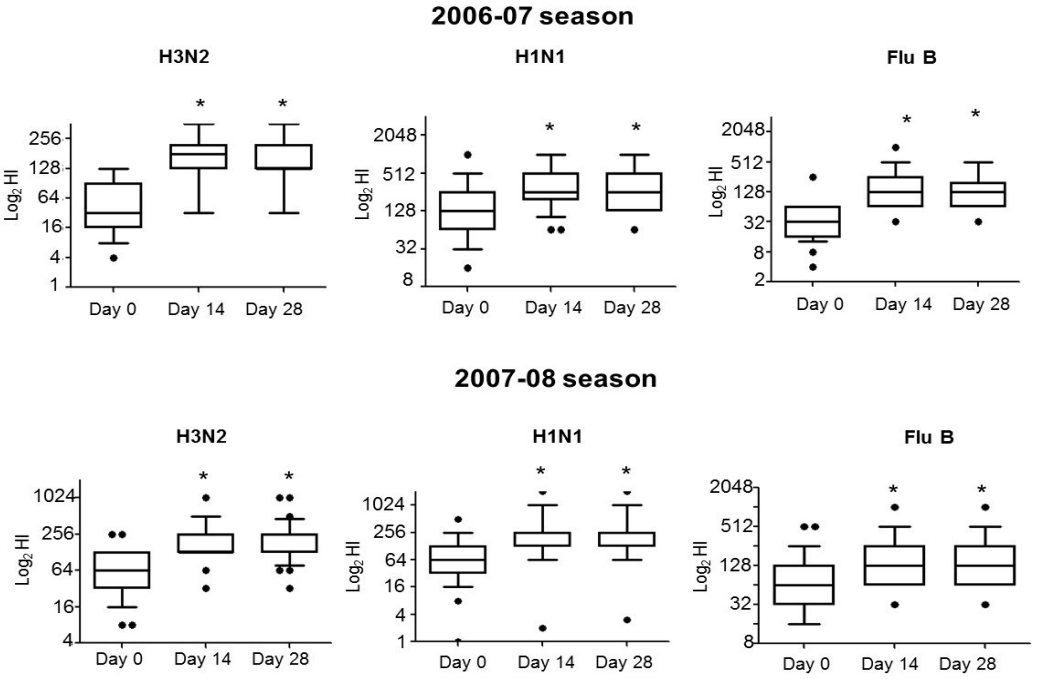
†p-values for differences in proportions calculated using Fischer's exact chi square test statistic; p-values for differences in means calculated using a t test statistic. Note: Due to n=10 subjects contributing to both seasons, these p-values are conservative estimates.

2.4.2 Serum antibody responses to seasonal vaccination

Induction of serum antibody responses is the current gold standard for evaluating influenza vaccines (175). Therefore, we first examined the serum antibody response to seasonal TIV and LAIV using the standard hemagglutination inhibition assay (HI). As shown in **Fig 2.1a**, TIV induced a significant increase in antibody titers at day 14 and day 28 post-vaccination during the 2006-07 and the 2007-08 influenza seasons. In contrast, the serum antibody response to LAIV was substantially lower than that of TIV (**Fig 2.1b**). As shown in **Table 2.2**, the sero-response rate, defined as the percentage of vaccine recipients who have a ≥4-fold increase in antibody titers post-vaccination (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074786.htm and http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074794.htm) at day 14 and day 28 post-vaccination was significantly greater in recipients of TIV compared to LAIV. No statistically significant differences were observed in the sero-response rates between male and female participants (data not shown). These data are in agreement with previous studies (162,176) suggesting that the vaccines used in this study induced antibody responses similar to those described previously.

Figure 2.1 Serum antibody response to seasonal TIV and LAIV

a. TIV



b. LAIV

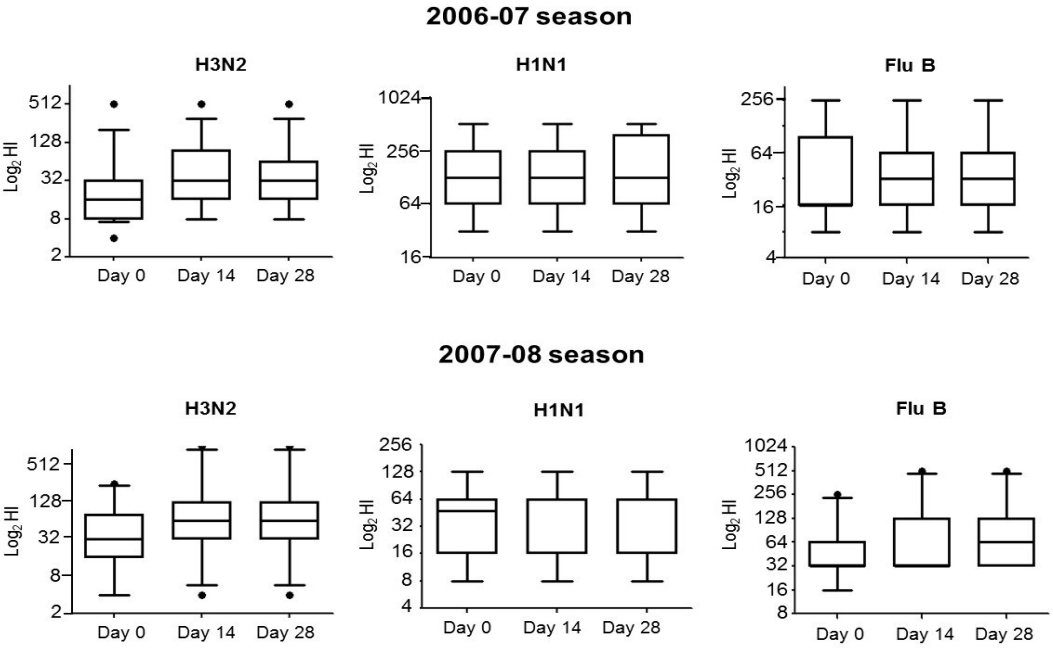


Fig 2.1. Serum antibody titers against influenza vaccine strains were determined by standard hemagglutination inhibition assay (HI). Antibody titers were determined prior to vaccination (day 0) and again at day 14 and day 28 post-vaccination with (**a**) TIV and (**b**) LAIV during the 2006-07 as well as the 2007-08 season. *p<0.05, student t test.

Table 2.2: Sero-response† rates at day 14 and day 28 by season, vaccine type and vaccine strain

	2006-07			2007-08		
Influenza strain	**TIV** N=25	**LAIV** N=17	**p-value‡**	**TIV** N=31	**LAIV** N=21	**p-value‡**
Day 14						
H1N1	36%	0%	**<0.01**	42%	0%	**<0.01**
H3N2	72%	12%	**<0.01**	48%	19%	**0.04**
Flu B	56%	0%	**<0.01**	23%	0%	**0.03**
Day 28						
H1N1	36%	0%	**<0.01**	45%	0%	**<0.01**
H3N2	68%	18%	**<0.01**	52%	29%	0.08
Flu B	56%	12%	**<0.01**	23%	5%	0.12

†Sero-response is defined as a ≥4- fold increase in titers from day 0. ‡P-value determined using Fischer's exact chi square test statistic.

2.4.3 Serum cytokine response to seasonal vaccination

To determine the effect of vaccination type on serum cytokines, we quantified the levels of 10 cytokines (IFN-γ, IL-1β, IL-2, IL-4, IL-5, TNF-α, IL-8, IL-12p70, IL-13 and IL-10) in the serum of volunteers before (day 0) and at 14 and 28 days after vaccination with either TIV or LAIV. As expected, the cytokine values were not normally distributed, so we examined medians (instead of means) in our statistical analyses. Of the 10 cytokines measured, only IL-10, IL-8 and TNF-α were reliably detectable in at least 70%

of our cohort at all three time points (days 0, 14 and 28 post-vaccination). Due to the difficulty in interpreting data with a large number of undetectable values, we restricted further statistical analyses to these three cytokines.

Despite small difference in the demographics between the 2006-07 and 2007-08 seasons, when we compared the median values of the three cytokines at day 0, we found no differences between the 2006-07 and 2007-08 seasons (**Table 2.3**). Thus we combined the data by vaccine type over the two seasons. Surprisingly, among TIV recipients, the levels of IL-8 decreased by 40% ($p<0.01$) at day 14 and 33% ($p<0.01$) at day 28 compared to levels at day 0 (**Fig 2.2**). Similarly, TNF-α levels also decreased significantly at days 14 and 28, albeit to a lesser extent (13% and 9% at days 14 and 28, respectively, $p<0.05$) (**Fig 2.2**). This effect was not observed in those vaccinated with LAIV (data not shown). The levels of IL-10 remained unchanged from baseline levels at both 14 and 28 days post vaccination regardless of the vaccine type (TIV: 2.05 pg/ml at day 0 compared to 2.0 pg/ml and 2.09 pg/ml at days 14 and 28, respectively, $p>0.05$. LAIV: 2.07 pg/ml at day 0 compared to 2.0 pg/ml and 2.11 pg/ml at days 14 and 28, respectively, $p>0.05$)

Table 2.3: Serum cytokine levels at day 0 (baseline), by year and vaccine type

	2006-07		2007-08		
Cytokine	**% detectable‡**	**Median (pg/ml)**	**% detectable‡**	**Median (pg/ml)**	**p value †**
TIV					
IL-10	77%	2	97%	2.28	0.62
IL-8	100%	13.29	100%	10.44	0.41
TNF-α	100%	7.39	100%	6.77	0.71
LAIV					
IL-10	82%	2.01	95%	2.13	1.11
IL-8	100%	10.65	100%	7.8	0.16
TNF-α	100%	8.49	100%	6.12	0.23

‡ Undetectable results were assigned a value equal to the lower limit of detection and included in the median calculations. † p-values for the differences in medians comparing 2006-07 and 2007-08 seasons were calculated using the Wilcoxon signed rank test

Figure 2.2 Serum cytokine levels at day 0, day 14 and 28 in TIV recipients

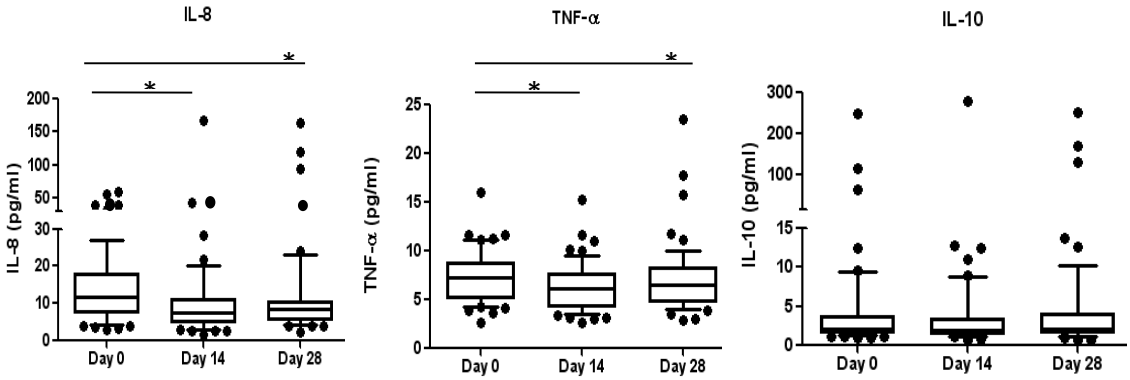


Fig 2.2. Serum cytokines were measured in serum of vaccine recipients prior to vaccination (Day 0) and at days 14 and 28 days post-vaccination. * $p < 0.05$, Wilcoxon rank sign test of medians using paired differences was used to determine the p-value.

2.4.4 Correlation between serum cytokine profiles and antibody responses

We next determined if the changes in IL-8 and TNF-α concentrations after vaccination correlated to "vaccine take" as measured by sero-response (175). However, no significant correlation could be found between sero-response at day 14 and day 28 and cytokine changes in TIV recipients in our cohort (data not shown).

Finally, to determine if baseline cytokine levels can predict sero-response, we compared median values of serum cytokines at day 0 between sero-responders and non-responders. Due to the poor sero-response rate in LAIV recipients (**Table 2.3**), we restricted our analysis to TIV recipients. There was a borderline statistically significant difference in the median baseline levels of IL-8 between sero-responders and non-responders (to any of the three influenza strains) at day 14 (**Table 2.4**). The median value of IL-8 was higher for sero-responders compared to non-responders (13.89pg/ml compared to 9.01pg/ml, p=0.05).

Table 2.4: Comparison of median cytokine levels at day 0 among sero-responders and non-responders in TIV recipients (2006-07 and 2007-08 season participants are combined)

Cytokine	Non-responders† (pg/ml)	Sero-responders† (pg/ml)	p-value‡
IL-10	2.16	2.01	0.90
IL-8	9.01	13.89	**0.05**
TNF-α	6.06	7.39	0.56

†Sero-response against any of the three vaccine strains. Sero-response is a ≥4-fold increase in titers from day 0 to day 14. ‡p-value comparing median cytokine value between responders and non-responders was determined using a Wilcoxon rank signed test.

2.5 Discussion

Vaccination remains one of the most effective means to control the spread of infectious diseases (177). However, little is known about the immunologic mechanisms which underlie effective vaccine strategies. In designing new vaccines against influenza as well as other infectious diseases such as malaria and HIV, understanding host factors that shape the development of protective immunity are key (178,179). Changes in cytokine profiles after infection have often provided important insight into the mechanisms mediating protective immunity to re-infection (167,168). Therefore, in this study, we sought to determine if serum cytokines could be used as a tool to better understand the immune response to seasonal influenza vaccination.

Serum anti-HA titers, measured by the hemagglutination inhibition assay (HI titers) are the gold standard for measuring influenza vaccine efficacy and, a greater than 4-fold increase in serum anti-HA titers is deemed a sero-response (157,175). Several factors have been shown to affect the sero-response rates in vaccine recipients including presence of circulating antibodies at the time of vaccination (176). Previous studies indicate that in individuals with pre-existing antibodies, the humoral response to TIV and LAIV is significantly lower than in individuals without pre-existing antibodies (164,176,180,181). Since we did not pre-select study participants based on sero-negativity to vaccine strains we found that the majority (>90%) of vaccine recipients had pre-existing antibodies at baseline to all of the vaccine strains included in both season's vaccines as shown in Table 2.1 (individuals with an HI titer of > 8 were deemed sero-positive as previously reported (180)). The lower sero-response rates reported in this study are consistent with sero-response rates reported in other seasonal influenza vaccine studies in healthy volunteers who were not pre-selected based on sero-

negativity to vaccine strains (162,181,182). One could speculate that circulating anti-HA antibodies at the time of vaccination could form complexes with HA in the vaccines, thereby reducing the amount of antigen available for stimulating naïve and/or memory B cells, thereby impacting the serum antibody response. In support of this hypothesis, Sasaki S, et al., demonstrated an inverse relationship between circulating anti-HA antibodies and peripheral effector B cell responses, at least to TIV (176). It would be interesting in future studies to determine if the presence of pre-existing antibodies negatively correlates with vaccine effectiveness.

In thus study we detected a substantially higher sero-response rate in recipients of TIV compared to LAIV corroborating findings from other studies (183,184). Despite the poor sero-response rates in LAIV recipients, both vaccines have been shown to be equally efficacious in preventing infection against homologous strains of virus (183-185) . This raises the possibility that immune mechanisms, other than serum antibodies, may contribute to protection, especially after vaccination with LAIV. In line with this, studies have shown that, in addition to serum IgG antibodies, induction of mucosal IgA antibodies also correlate with protection against influenza infection (163,186). Importantly, it has been shown that LAIV induces an increase in such mucosal IgA antibodies in vaccine recipients (164,182). In fact, such mucosal responses were more frequent in recipients of LAIV than TIV, suggesting that the two vaccines may stimulate different antibody compartments (185). In addition, the impact of either seasonal vaccine on other immunological parameters such as T cell responses are not well understood and warrant further study. This will be the focus of the next chapter of this thesis.

Previous studies characterizing cytokine changes following vaccination have typically focused on early time points immediately following vaccination during innate recognition (187). While rapid changes in cytokines can provide important clues regarding the

activation of different innate cell subsets, their correlation with adaptive immunity is not clear. Studies in mice suggest that the levels of cytokines at the peak of the adaptive immune response (typically between days 7-14 post infection) correlate with the type of T helper and B cell response to influenza vaccination (188,189). Accordingly, in this study, we examined the sustained cytokine levels at the peak of the adaptive immune response to gain insight into mechanisms of adaptive immunity. We found that immunization with TIV, but not LAIV, was associated with a significant decrease in the serum levels of IL-8 and TNF-α at 14 and 28 days post-vaccination. IL-8 and TNF-α are potent pro-inflammatory mediators that contribute to host protection against influenza as well as other infectious pathogens (190-192). During the early stages of the immune response to pathogens including influenza, TNF-α (Y. Chen and J. Bream, manuscript in preparation) and IL-8 are actively secreted by innate mononuclear cells as well as endothelial cells (193). IL-8 in particular, serves as an important chemoattractant and recruits other inflammatory cells including neutrophils and T cells to the site of infection/vaccination (194). The decrease in the serum levels of IL-8 and TNF-α at later time points after vaccination could reflect cytokine uptake or redistribution of immune cells to other compartments. Interestingly, a similar decrease in other soluble mediators of inflammation including MIP-1β, MIP-1α and IFN-γ was observed during the acute phase of seasonal and pandemic influenza infections (195).

In contrast to TIV, we observed no significant changes in cytokines at either time-point after vaccination with LAIV. Since LAIV is administered through an intranasal route, it is possible that, like antibodies (183,196), cytokine responses may also be contained within the local mucosa and not readily detectable in the periphery. This idea is in agreement with previous studies that reported changes in cytokine profiles in nasal wash

but not serum of animals and humans infected or vaccinated with influenza and RSV (197-199). Measuring nasal wash cytokines may, therefore, be more instructive in understanding mechanisms of immune activation following administration of LAIV and other mucosally-delivered vaccines. Alternatively, and as previously reported, it is possible that the kinetics of the cytokine response differs in TIV and LAIV recipients and that our study missed the peak in the peripheral cytokine response after LAIV administration (199).

Using a systems biology approach, other groups have shown that early immune signatures can predict adaptive responses to immunization, including seasonal influenza vaccination (187,200). Identifying early markers of immunogenicity might help in early identification of non-responders, which could prove valuable from a public health standpoint. We found that sero-responders to TIV had modestly higher baseline levels of IL-8 compared to non-responders ($p=0.05$, Table 5). Consistent with this finding, several other groups also reported higher baseline levels of IL-8 in patients who respond better to BCG vaccination in the prevention of recurrence of bladder tumors (201,202). However, given the weak association between baseline levels of IL-8 and sero-response in our cohort, it is too soon to speculate on the significance of this finding.

There are some caveats to this study that should be noted. Firstly, the sample size is relatively small and consisted only of healthy adults. Thus, age or smaller effects may have been missed. In addition, the small, but consistent, absolute change in IL-8 and TNF-α levels after TIV draws into question the biological meaning of small shifts in cytokine amounts. While the biological significance of such small changes has yet to be determined, another study has shown that even small doses of recombinant cytokines and chemokines, administered *in vivo*, can impact signaling pathways in peripheral cells

(203). Finally, changes in serum cytokines/ chemokines that may have occurred immediately following vaccination with either TIV or LAIV were likely missed in this study and warrant further study.

In conclusion, in this study we report for the first time that the killed, parenteral (TIV) but not a live, mucosal (LAIV) seasonal influenza vaccine has the capacity to alter the balance of peripheral cytokines up to 4 weeks post-vaccination. This study serves as a proof of principle that serum cytokine changes following seasonal influenza vaccination can be detected. Their significance with respective to the induction of protective immunity warrants further investigation. Our data also suggest that baseline IL-8 levels may impact the development of the adaptive immune response to TIV. It will be important for future studies to determine how changes in cytokine profiles may relate to changes in immune cell activation to provide another tool to dissect the cellular and molecular mechanisms of vaccine-induced immunity. Furthermore, such studies could prove valuable in understanding host factors which regulate the immune response to vaccines and natural infection.

CHAPTER 3: Heterotypic T cell responses to seasonal influenza vaccination in humans

3.1 Abstract

Mounting evidence suggests that seasonal influenza vaccines may not be as efficacious as once thought which underscores the need to better understand vaccine-induced immunity. Although antibodies provide influenza strain-specific protection against infection, the role of cell mediated immune responses in host defense against influenza are being increasingly recognized. This is of particular importance considering the ongoing threat of emerging pandemic influenza strains. Using a cohort of vaccinees from the 2006-07 season we examined the capacity of the trivalent influenza vaccine (TIV) and the live attenuated influenza vaccine (LAIV) to induce homologous and heterologous T cell responses. We used a combination of circulating and novel influenza viruses and select peptide pools to assess influenza-specific T cell responses. We found that prior to vaccination, $CD4^+$ and $CD8^+$ T cells, unlike antibodies, were broadly cross-reactive to whole, heterotypic influenza A viruses. Interestingly, vaccination with TIV and LAIV, induced significant increases in the frequency of homotypic and heterosubtypic influenza-specific $CD4^+$ T cells. This increase in total T cell frequency was also accompanied by a change in T cell quality, at least in recipients of TIV. Further studies are required to determine if these changes in total T cell magnitude and/or quality against seasonal and newly emergent influenza strains after vaccination will serve to minimize disease in a clinical setting.

3.2 Introduction

Influenza is a respiratory pathogen that continues to pose a significant public health risk resulting in 3 to 5 million cases of severe disease worldwide every year (World Health Organization WHO factsheet 211:influenza; 2003) and between 17,000-51,000 deaths in the United States every year (3). In addition to seasonal epidemics, newly emerging influenza strains (such as H5N1 and H7N9) remain a serious threat to global public health. With an estimated mortality rate of 70% and 30% for H5N1 and H7N9 infections, respectively (204), the spread of such highly pathogenic strains in the human population could have catastrophic effects on human health and world economy.

Vaccination remains the most effective means to reduce the disease burden and economic impact of influenza (205). In the United States there are two types of approved seasonal influenza vaccines: the Trivalent Inactivated Influenza Vaccine (TIV) and the Live Attenuated Influenza Vaccine (LAIV). Influenza vaccines are generally thought to confer protection by the induction of antibodies against the two major surface proteins, namely Hemagglutinin (HA) and Neuraminidase (NA) (206). Due to continual antigenic drift in HA and NA, the seasonal vaccines are reformulated every year to match circulating strains. When the vaccine and circulating strains are well matched, the seasonal vaccines were previously thought to be between 90-100% effective against seasonal infections (130,139). However, their true effectiveness has recently been debated with many studies reporting effectiveness of only 50% in healthy adults and as low as 6% in vulnerable population such as young children and the elderly, even during years of good antigenic match (123,142). In years of mismatch or in the event of an emergence of a new pandemic, as in 2009, the efficacy of the current vaccines in preventing infection is thought to be even lower (207,208). In addition, recent studies have reported a decline in antibody levels just one month after vaccination resulting in a

reduction in vaccine effectiveness over time (209). Taken together, these data highlight some major limitations and drawbacks in the current vaccines and underscore the need for more effective strategies.

To this end, there has been a substantial increase in vaccine discovery and development efforts, as outlined in section 1.3.5 (206). The development of more effective vaccines that can induce long lived immunological memory across various influenza subtypes, or the so-called “universal” influenza vaccine is critical to controlling disease burden. However, the rational design and development of such new vaccines has been slow and challenging, in part because little is understood about the immunological underpinnings of successful vaccination (210). In fact, LAIV still has no accepted correlate of protection.

The vast majority of past work has focused on the ability of influenza vaccines to induce strain-specific serum anti-HA antibodies (175) with little regard to mechanisms that drive optimum antibody production or cross-reactive immunity (162,164). An accumulating body of evidence suggests that T cells may contribute to both of these processes. Unlike B cells, T cell recognition of influenza is not restricted to surface proteins and instead can mount responses to the internal proteins, which are highly conserved across different influenza strains (80,211,212). Accordingly memory T cells established by seasonal infections have been shown to recognize novel influenza viruses and are thought to be important mediators of heterosubtypic immunity, at least in animal models. In humans, this idea has only recently been reinforced by studies which identify a direct link between memory T cells and protection from severe disease. For instance, experimental challenge with influenza demonstrated that pre-existing memory CD4 T cells (induced either through vaccination or natural infection) could mediate viral clearance and recovery from infection, even in the absence of detectable serum

antibodies (78). Similarly, another study of over 300 individuals showed that pre-existing memory CD8 T cells were able to protect from severe disease during the 2009 pandemic (81). Thus T cells may be able to provide partial protection against novel influenza viruses. In addition to contributing to direct clearance of infected cells, T cell responses have also been shown to be crucial for the induction and maintenance of long-lived B cell memory and high affinity antibody production (67,68,213), which is of course of great interest from a vaccination standpoint.

The commercially available influenza vaccines offer a platform to study the development of T effector as well as memory responses in humans. The seasonal influenza vaccines are additionally unique because they also allow for comparisons of different routes of immunization and/or vaccine formulations on T cell immunity. This information could be invaluable in guiding future vaccination strategies not just against influenza but also other pathogens for which induction of T cell immunity is likely to be important, such as HIV or tuberculosis (206,210). A major hurdle in the assessment of T cell responses is that currently there are no accepted standard assays (210,214). The added complexity of T cell heterogeneity based on functional (ability to secrete multiple effector molecules including IFN-γ, TNF-α and IL-2) and memory phenotype (based on expression of tissue homing markers into central memory and effector memory) further confounds this issue (215,216). Variations in cell and reagent handling, stimulation protocols and functional and phenotypic measurements has made cross-study comparisons impossible. The capacity of influenza vaccines to induce T cell responses has been somewhat contentious, in large part due to this variability (182,217-220). In this study, we developed a novel flow cytometry-based lyophilized antibody/antigen platform for the high-throughput characterization of influenza-specific T cells. We then used this platform to address the capacity of the two seasonal influenza vaccines (TIV

and LAIV) to induce homotypic CD4 and CD8 T cell responses against strains contained in the vaccine as well heterotypic T cells responses to divergent strains including the 2009 pandemic H1N1 strain.

3.3 Methods

Study design and human subjects

Forty two participants between the ages of 18 and 49 were recruited in the fall of the 2006-07 influenza season between October and November. Participants were screened by questionnaire at each visit to assess health history including recent acute illness. Volunteers were excluded from the study if they reported to be immuno-compromised due to chronic illness or medication and there were no reports of recent acute illness during the study visits. All volunteers provided written informed consent and choose their vaccine type: LAIV (Flumist, Medimmune) or TIV (Fluzone, Aventis Pasteur). A total of 17 participants received LAIV and another 25 received TIV, of whom 17 were randomly selected to be included in this study. Immediately prior to vaccination (day 0), a venous blood specimen of approximately 50 ml was collected. Volunteers were then immunized following current guidelines for influenza vaccination. Participants provided additional blood samples at 14 (day 14) and 28 days (day 28) after vaccination. Serum was harvested from blood samples using serum separator tubes (BD Bioscience, San Jose, CA) and stored at -80˚C. The 2006-07 seasonal vaccines contained A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2) and B/Malaysia/2506/2004.

PBMC isolation and cryopreservation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using Ficoll-Paque density gradient centrifugation. Briefly, whole blood gently layered on top of Ficoll-Paque in a 50ml conical tube. Cells were then centrifuged at 2400rpm for 20 minutes at room temperature with brake. After centrifugation, the interface containing mononuclear cells was carefully transferred using a 10ml pipette to a new conical tube. Transferred mononuclear cells were diluted in distilled phosphate buffered saline

(DPBS) and centrifuged twice at 1500rpm for 15 minutes at room temperature. Washed PBMCs were frozen at a concentration of $1x10^7$/ml in freezing media (20%FBS+80% RPMI with penicillin and streptomycin)

Influenza viruses, peptides and antibodies

Influenza viral strains used in this study: A/Wisconsin/67/2005 (H3N2), A/New Caledonia/20/1999 (vH1N1), A/Solomon Islands/3/2006 (sH1N1), A/California/04/2009 (pH1N1) and B/Malaysia/2506/2004 (Flu B), were obtained from the CDC. The negative-sense RNA virus vesicular stomatitis virus (VSV) was kindly provided by Dr. Andrew Pekosz from the Johns Hopkins School of Public Health and was used as a negative control. All viruses were propagated in Madin-Darby canine kidney (MDCK) cells and purified using a 20% sucrose cushion. Viruses were then inactivated using 0.05% β-propiolactone (BPL) treatment overnight at 4˚C. Samples were then heated at 37˚C for 2 hours to inactivate the compound. Virus inactivation was confirmed by the lack of cytopathic effects (CPE) on MDCK cells.

Individual peptides from influenza proteins HA (NR-9472), M1 (NR-2613), NP (NR-2611) and PB1 (NR-2617) were obtained from the NIH Biodefense and Emerging Infections Research Repository, NIAID, NIH. Peptides were suspended in DMSO and pooled to generate peptide pools for each protein. Peptides were 13- to 17-mers with 7 to 12 amino acid overlaps. The HA, M1, NP and PB1 pools consisted of 94, 41, 82 and 126 peptides respectively. *Staphylococcal enterotoxin* B (S4881, Sigma Aldrich) was used at a concentration of 1 µg/ml as a positive control for antigen stimulation.

Hemagglutination inhibition assay (HI)

HI titers were measured in serum samples for all three vaccine strains in each season as described in the previous chapter. Influenza virus strains A/New Caledonia/20/1999 (H1N1), A/Wisconsin/67/2005 (H3N2), B/Malaysia/2506/2004 and A/ Solomon Islands/3/2006 (H1N1) were obtained from the CDC. Serum samples were pretreated with receptor destroying enzyme (Lonza Inc., Walkersville, MD) overnight at 37˚C. Samples were then treated with Sodium Citrate (1.6%) and heated at 56˚C for 45 minutes. The hemagglutination inhibition (HI) assay was performed as described previously (183). Briefly, 25 µl of specific influenza virus (4 HA units) was added to 25 µl of serum serially diluted in 2-fold dilutions till 1:2048 in PBS and incubated for 1 hour at room temperature. 50 µl of 0.5% chicken red blood cells (for influenza A/ H1N1 and influenza B) and 0.5% turkey red blood cells (for influenza A/ H3N2 and A/ pH1N1) was then added and incubated for a minimum of 30 minutes before reading the HI activity. All erythrocytes were obtained from CBT farms (city, state). The HI antibody titer for a given sample was defined as the reciprocal of the last dilution at which there was complete inhibition of HA activity. A titer of 2 was assigned to all samples in which the first dilution (1:4) was negative.

Lyophilized reagent plates for stimulation and staining

Stimulation plates were formulated with individual peptide pools at a concentration of 1.7 µg/peptide and Brefeldin A (BFA) at a concentration of 10 µg/ml or purified inactivated viruses (Multiplicity of infection =1) without BFA and then lyophilized into poly-propylene V-bottom 96-well plates. Similarly, staining plates were formulated with appropriate antibody mixtures in appropriate wells and lyophilized. Two antibody mixtures were used: The surface staining cocktail consisted of: CD3-APC-H7, CD4-

PerCPCy5.5, CD8-V500, CCR7-PE, CD45RA-PECy7 and CD28-APC. The intracellular staining cocktail consisted of IFN-γ -V450, IL-2-FITC and TNF-α-Alexa 700. All antibodies were obtained from BD Biosciences.

Stimulation for intracellular cytokine assays

Cryopreserved PBMCs were thawed and rested for four hours at 37ºC. Rested cells were added to each well of the lyophilized stimulation plates containing peptide pools or whole inactivated viruses at a concentration of $5x10^6$ cells/ml and mixed well with micropipetter to reconstitute the lyophilized pellet in a total volume of 100 µl. Control media containing BFA was used for background subtraction. For peptide stimulations, cells were stimulated for 6 hours at 37°C and then transferred to staining plates. For whole virus stimulations, PBMCs were added to wells containing whole virus in 100 µl of incomplete media lacking FBS. Cells were incubated for 1 hour at 37°C and then reconstituted with 10% FBS. Cells were then incubated for another 17 hours and BFA was added for the last 6 hours, following which the cells were transferred to staining plates.

Staining for intracellular cytokine assays

Stimulated cells were washed twice in 1x PBS and then stained with LIVE/DEAD fixable stain (Invitrogen, L34959) as per manufacturer's instructions. Surface and intracellular staining was then performed on cells as described previously with minor modifications (221). Briefly, staining plates containing the surface staining cocktail were hydrated in wash buffer (PBS +0.5% BSA) and then added to the stimulated cells. Plates were incubated at room temperature for 30 minutes and then washed once. Cells were

then incubated with FACS lysing solution (BD Biosciences) for 15 minutes at room temperature. Plates were then frozen at -80°C before further processing. Frozen plates were thawed at 37°C and washed once in wash buffer. Permeabilizing Solution 2 (BD Biosciences) was then added and plates were incubated for 10 minutes at room temperature. Plates were washed twice in wash buffer and finally the rehydrated intracellular staining antibodies were added and incubated for 1 hour at room temperature. Plates were then washed twice, suspended in 1x PBS and then acquired on a BD LSR II flow cytometer and analyzed using FlowJo (Tree Star).

Statistical analysis

Baseline comparisons of T cell and antibody responses between viruses were tested using the Kruskal-Wallis test. Changes in cytokine secreting CD4 and CD8 T cell frequencies pre- and post-vaccination were tested using Wilcoxon signed rank test. Statistical comparison of pies were performed in SPICE software using 10,000 permutations (222).

3.4 Results

3.4.1 Assay optimization and lyoplate generation

Reagent lyophilization can potentially allow for the standardization of reagent handling and protocols across multiple laboratories (223-225). However, lyophilized reagent plates (henceforth referred to as lyoplate) have not previously been used for whole virus stimulations. Therefore, we first optimized the conditions for CD4 and CD8 T cell stimulation using whole inactivated viruses. We tested three different methods of virus inactivation namely, β-propiolactone, heat inactivation at 56ºC and UV inactivation. We found that stimulation of T cells with virus inactivated with β-propiolactone was closest to live virus stimulations in terms of IFN-γ and IL-2 production (data not shown). In addition, virus inactivation was most consistent using β-propiolactone. Therefore all viruses were inactivated with β-propiolactone in this study. Other parameters tested included concentrations of virus and durations of stimulation (data not shown).

To ensure that lyophilization of antigen and/or antibody did not affect T cell stimulation or staining, the standard soluble assay was carried out side-by-side with lyophilized reagent plates. **Fig 3.1** shows representative pre-vaccination samples stimulated with A/New Caledonia/20/1999 *ex vivo* either via the standard soluble assay or via lyophilized reagents (hereby referred to as lyoplate). The two assays were highly comparable in terms of staining as well as stimulation as evidenced by the percentage of cytokine producing CD4 (**Fig 3.1a**) and CD8 T cells (**Fig 3.1b**). Data represented in the rest of this chapter was therefore generated using lyoplates.

Figure 3.1 Comparison of soluble and lyophilized platforms

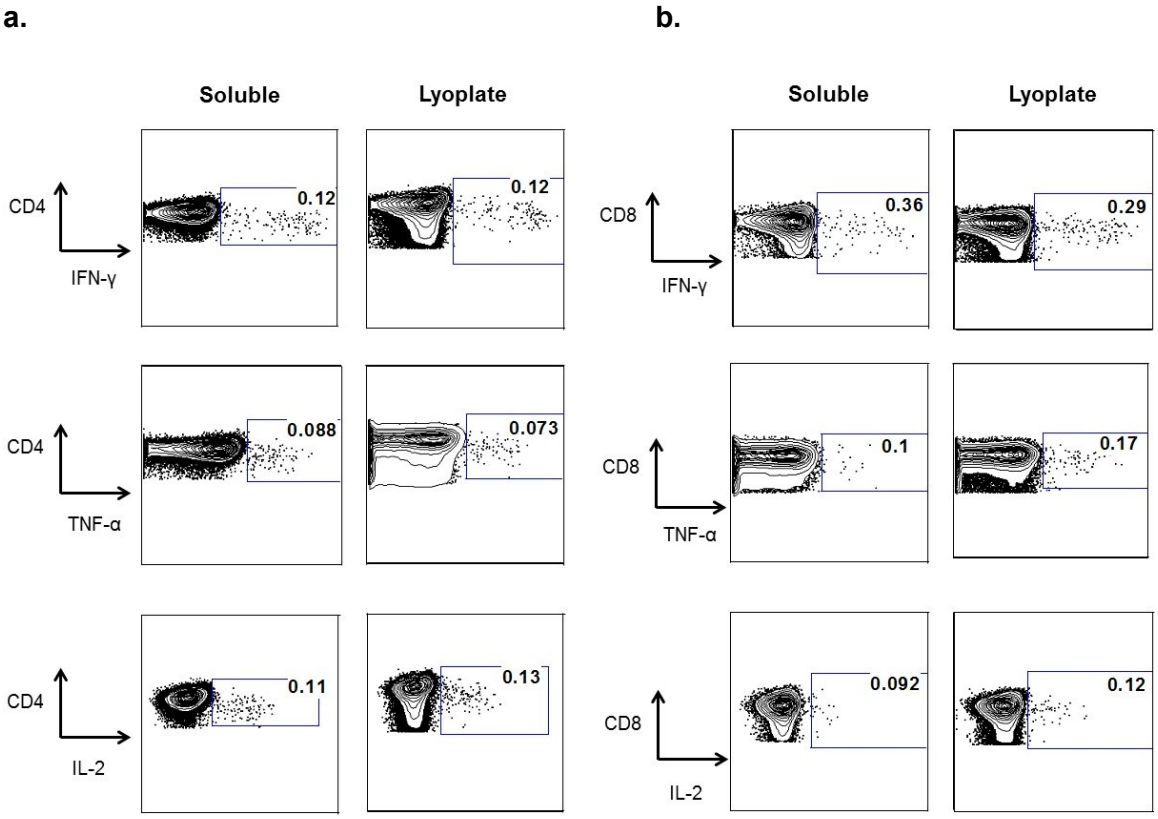


Fig 3.1. PBMCs were stimulated and stained using soluble reagents or pre-configured lyophilized reagents (lyoplates). Representative plots from a pre-vaccination sample stimulated with A/New Caledonia is shown for CD4 (**a**) and CD8 (**b**) T cells stained for IFN-γ, TNF-α and IL-2.

3.4.2 Cross-reactivity of influenza-specific antibodies prior to vaccination

Serum antibodies are an established correlate of protection against influenza and the gold standard for vaccine effectiveness (at least for TIV) (85). Therefore, we first examined the extent of antibody cross-reactivity to circulating and novel influenza strains by measuring anti-HA antibodies in the serum of vaccine recipients prior to seasonal vaccination. Consistent with previous exposure, all participants had detectable anti- HA antibodies (HI titer>8 as indicated by the dashed line, **Fig 3.2**) against strains that were in circulation prior and during sample collection (A/New Caledonia/20/1999- vH1N1,

A/Wisconsin/67/2005- H3N2 and B/Malaysia/2506/2004- Flu B)(226). In addition, we measured antibody titers against an antigenic variant of A/New Caledonia/20/99, namely A/Solomon Islands/3/2006-sH1N1, which had widespread activity in the United States only during the 2007-8 season(227), and the 2009 pandemic H1N1 (A/California/4/2009)-pH1N1. While the circulating strain (vH1N1) and seasonal drift variant (sH1N1) share 98% sequence identity in the HA protein, antibody titers against sH1N1 were significantly lower than vH1N1 (HI titer of 128 against vH1N1 compared to 32 against sH1N1, $p<0.001$). These data suggest poor conservation in the antibody binding epitopes in HA, even between closely related strains. Not surprisingly, titers against the pandemic H1N1 strain (pH1N1), which only shares 75% sequence identity with the circulating H1N1 HA, were the lowest amongst all influenza strains tested (HI titer of 16).

Figure 3.2 Baseline (day 0) antibody titers against influenza strains

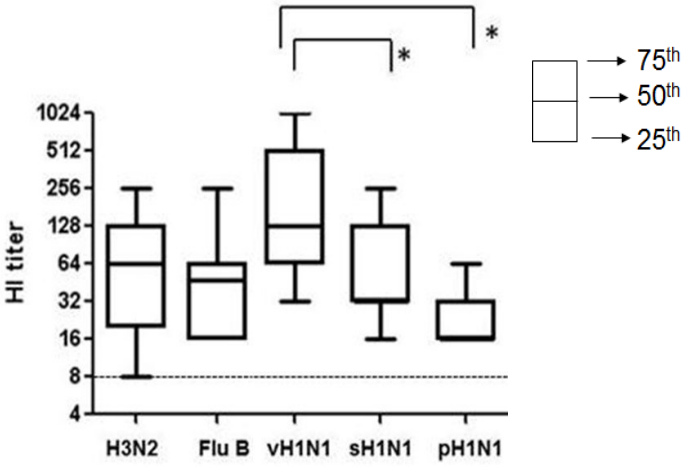


Fig 3.2. Serum anti-HA antibody titers to various influenza strains prior to vaccination was determined using the hemagglutination inhibition assay (HI) (TIV and LAIV groups were combined at baseline; N=42). The dashed line indicates seronegativity (HI<8). *p<0.001, Kruskal-Wallis test.

3.4.3 Cross-reactivity of influenza-specific T cells prior to vaccination

Next, we examined the extent of T cell cross-reactivity against the same influenza strains. Cryopreserved PBMCs were stimulated with lyophilized whole, β-propiolactone inactivated influenza virus and the frequency of total cytokine (IFN-γ, IL-2 or TNF-α or any combination of the three) producing CD4 and CD8 T cells was evaluated as described below. A gating tree from a representative pre-vaccination sample stimulated *ex vivo* with A/New Caledonia/20/1999 is shown in **Fig 3.3**. As described previously (170), within the gated $CD4^+$ and $CD8^+$ T cell population, cells were separated into IFN-γ^+ and IFN-γ^- cells and further assessed for the production of IL-2, TNF-α or both. This analysis revealed 7 functionally distinct populations of cells that make IFN-γ, IL-2, TNF-α or any combination of the three. Together, these seven populations comprise the frequency of total cytokine producing CD4 or CD8 T cells.

Similar to antibodies, most individuals (>95%) had detectable levels of CD4 (**Fig 3.4a**) and CD8 T (**Fig 3.4b**) cells against strains that were in circulation prior to sample collection (A/New Caledonia/20/1999-vH1N1, A/Wisconsin/67/2005-H3N2 and B/Malaysia/2506/2004-Flu B). However, in contrast to antibody titers, we detected significant CD4 and CD8 T cell cross-reactivity to antigenic variants, namely A/Solomon Islands/3/2006-sH1N1 and A/California/4/2009-pH1N1. As shown, CD4 and CD8 T cells against sH1N1 and pH1N1 were detected in the majority (>95%) of our participants prior to vaccination. In fact, the frequency of CD4 and CD8 T cells against pH1N1 and sH1N1 was comparable to that against the circulating H1N1 strain (vH1N1)($p>0.05$) and was higher than those against other circulating influenza strains (A/Wisconsin/67/2005-H3N2 and B/Malaysia/2506/2004-FluB).

While antibodies against influenza are predominantly directed against hemagglutinin (HA), we were able to detect CD4 and CD8 T cells against several internal proteins, with the highest frequency of CD4 and CD8 T cells directed against NP (**Fig 3.4c** and **d**). The ability of CD4 and CD8 T cells to recognize invariant epitopes derived from highly conserved internal proteins (75) is likely responsible for the high degree of cross-reactivity to antigenic variants observed in this and other studies (211).

Figure 3.3 Gating tree for functional characterization of influenza specific T cells

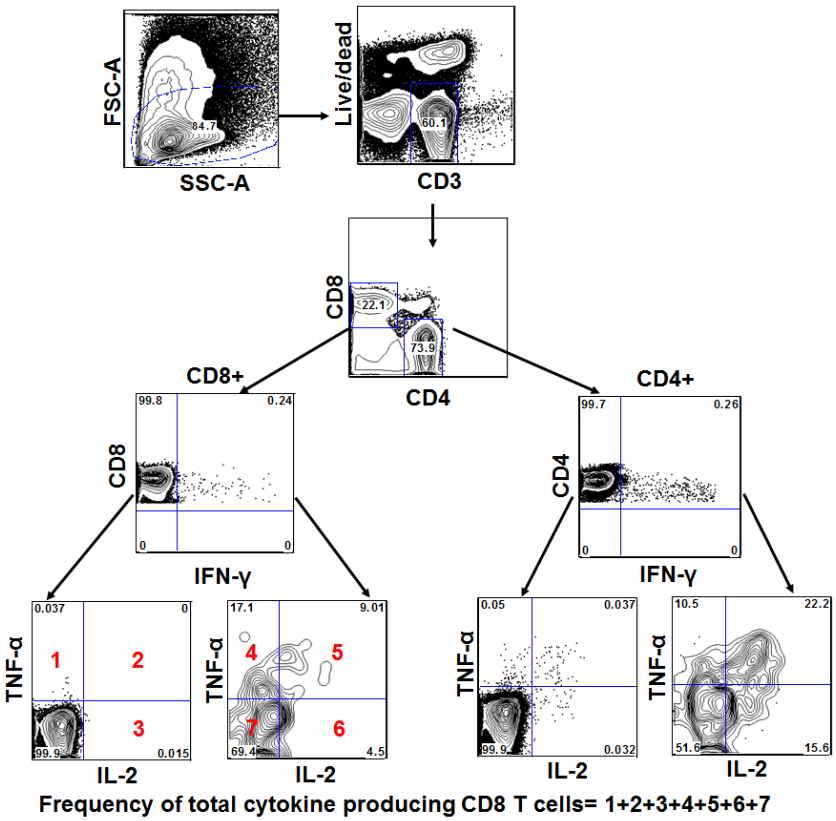


Fig 3.3. The gating strategy is shown for a representative pre-vaccination samples stimulated *ex vivo* with influenza strain A/New Caledonia/20/1999. Based on the expression of IFN-γ, TNF-α and IL-2, seven distinct functional populations of cytokine producing cells can be ascertained (numbered 1-7 in **Fig 3.3**). These seven populations together comprise the total cytokine producing population of $CD4^+$ and $CD8^+$ T cells

Figure 3.4 Baseline (day 0) T cell responses to influenza viruses

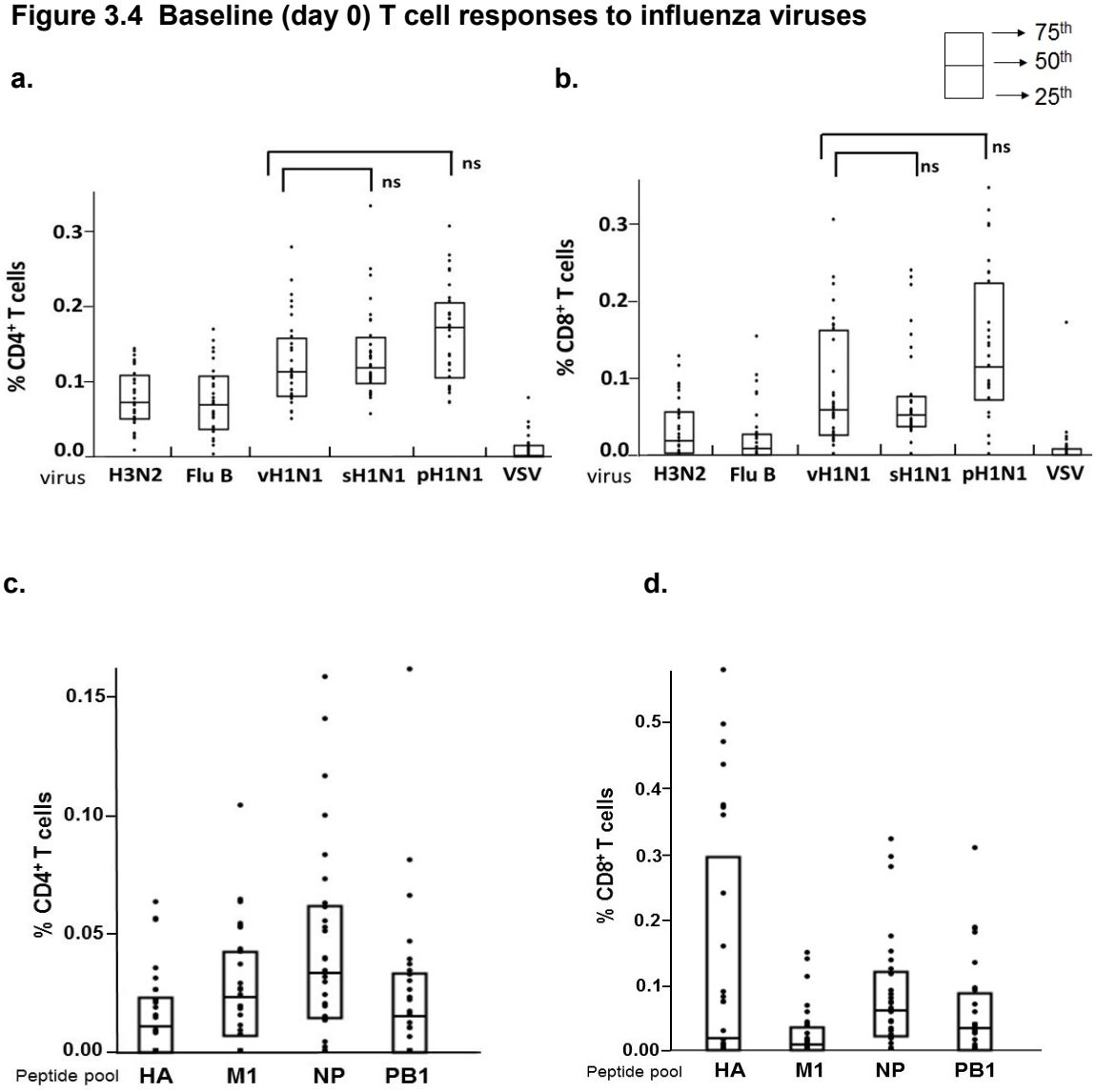


Fig 3.4. PBMCs were stimulated *ex vivo* with whole, β-propiolactone inactivated influenza viruses and vesicular stomatitis virus (VSV) as negative control. Frequency of virus-specific $CD4^+$ (**a**) and $CD8^+$ (**b**) T cells (comprising of IFN-γ, TNF-α, IL-2 or any combination of the three cytokines) was determined by multicolor flow cytometry. Additionally, PBMCs were also stimulated with individual peptide pools derived from influenza proteins and the frequency of antigen specific $CD4^+$ (**c**) and $CD8^+$ (**d**) T cells (comprising of IFN-γ, TNF-α, IL-2 or any combination of the three cytokines) was determined. Individual values are shown in dots. (TIV and LAIV groups were combined at baseline; N=42) *ns*, not significant, Kruskal-Wallis test.

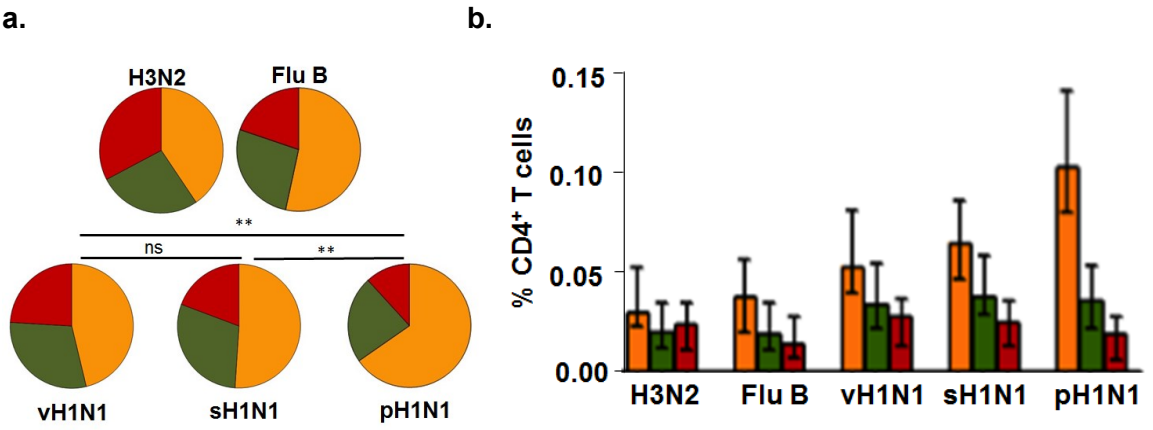
3.4.4 Functional and phenotypic characterization of influenza-specific T cells

After evaluating the total CD4 and CD8 T cell response to influenza in unvaccinated adults, we then evaluated the "quality" of the response as determined by the co-expression of IFN-γ, TNF-α and IL-2 (215). The quality of the T cell response refers to the functional capacity of T cells (in terms of cytokine secretion, expression of cytolytic markers, etc). T cells that can simultaneously express multiple effector functions have been previously shown to correlate with improved protection after vaccination and are therefore considered to have a better quality (59,228-231). To determine the "quality" of the CD4 and CD8 T cell response, we combined the 7 cytokine producing populations described above into 3 functional categories based on the number of cytokines that are simultaneously expressed (any one, any two and all three) (215). As shown in **Fig 3.5a and b**, virus-specific cytokine expression patterns in CD4 T cells were heterogeneous and consisted of single, double and triple cytokine producing populations. CD4 T cells making any one cytokine (IFN-γ, IL-2 or TNF-α) constituted approximately 40-50% of the total cytokine producing CD4 T cell population against influenza while double and triple producers accounted for 30% and 20% of the response respectively (**Fig 5a**). Interestingly, the CD4 T cell cytokine response against pH1N1 showed the least heterogeneity with close to 70% of cells making only one cytokine. In contrast, CD8 T cell responses against all viruses were largely restricted to single cytokine producers (accounting for approximately 70% of the total response) (**Fig 3.5c and d**). Once again, the CD8 T cell response to pH1N1 was more restrictive in terms of their cytokine secretion profiles with over 90% of the total CD8 T cell response to pH1N1 comprised of single cytokine producers compared to 70% against seasonal H1N1 viruses (**Fig 3.5c**).

In addition to qualitative differences, CD4 and CD8 T cells also possess distinct phenotypic profiles that maybe predictive of protective efficacy. A well-established model

of classification is based on the expression of CD45RA and CCR7 (216,232,233). Based on the expression of these surface markers, CD4 and CD8 T cells can be divided into naïve-like ($CD45RA^+CCR7^+$), central memory T_{CM} ($CD45RA^-CCR7^+$), effector memory T_{EM} ($CD45RA^-CCR7^-$) and terminal effector T_{TE} ($CD45RA^+CCR7^-$) populations. To determine the phenotype of cytokine producing cells, we included only those samples with > 30 cytokine+ events and cytokine+ frequencies >3x over background (222). CD45RA and CCR7 gates were set on total CD4 and CD8 T cells (**Fig 3.6a**) and then applied to cytokine+ CD4 and CD8 T cell populations. As shown in **Fig 3.6b** cytokine+ CD4 T cells against seasonal influenza strains were predominantly effector memory (T_{EM}) and central memory (T_{CM}) cells, constituting approximately 40% and 30% of the total response, respectively. Terminally differentiation effectors (T_{TE}) accounted for a small percentage of the total response (roughly 10%). Interestingly, a sizeable proportion of cytokine+ CD4 T cells (20%) were found to have a naïve-like phenotype. Similarly, cytokine+ CD8 T cells were predominantly T_{EM} (40%-50%) (**Fig 3.6c**). However, greater variability in surface phenotype in CD8 T cells against the different viruses was noted.

Figure 3.5 Quality of influenza-specific T cells at baseline (day 0)



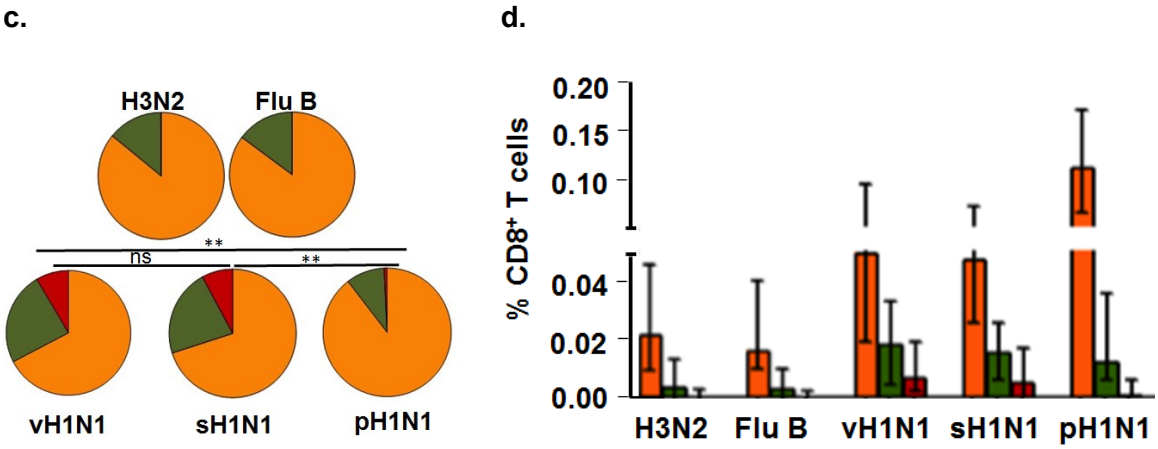
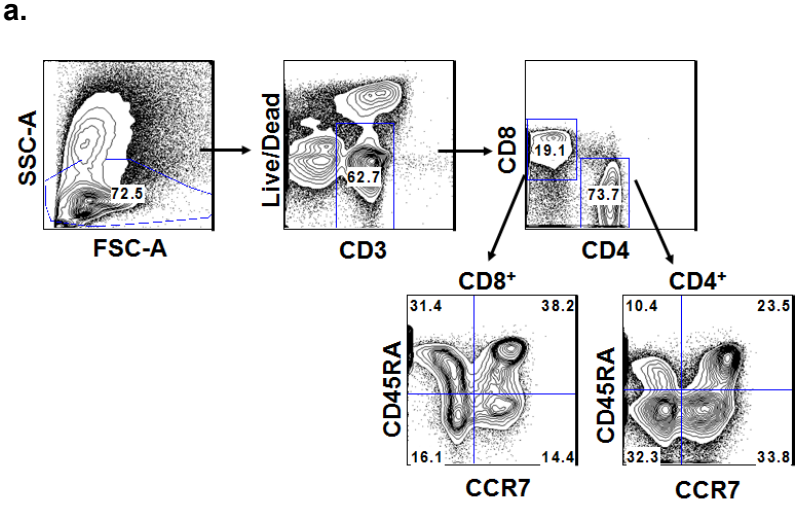


Fig 3.5. (**a**) The relative proportion of cells within total cytokine producing $CD4^+$ T cells that make any one (IFN-γ, TNF-α or IL-2), a combination of any two and all three cytokines was determined for each virus. (**b**)The frequency of $CD4^+$ T cells that are single ( ), double ( ) or triple cytokine ( ) positive. (**c**) The relative proportion of cells within total cytokine producing $CD8^+$ T cells that make any one (IFN-γ, TNF-α or IL-2), a combination of any two and all three cytokines (**d**) Th e frequency of single, double and triple cytokine positive $CD8^+$ T cells. TIV and LAIV groups were combined at baseline, (N=42). **p<0.0001. A/Wisconsin/67/2005-H3N2 and B/Malaysia/2506/2004-Flu B; A/New Caledonia/20/1999-vH1N1, A/Solomon Islands/3/2006-sH1N1 and A/California/4/2009-pH1N1. *

Figure 3.6 Phenotype of influenza-specific T cells at baseline (day 0)



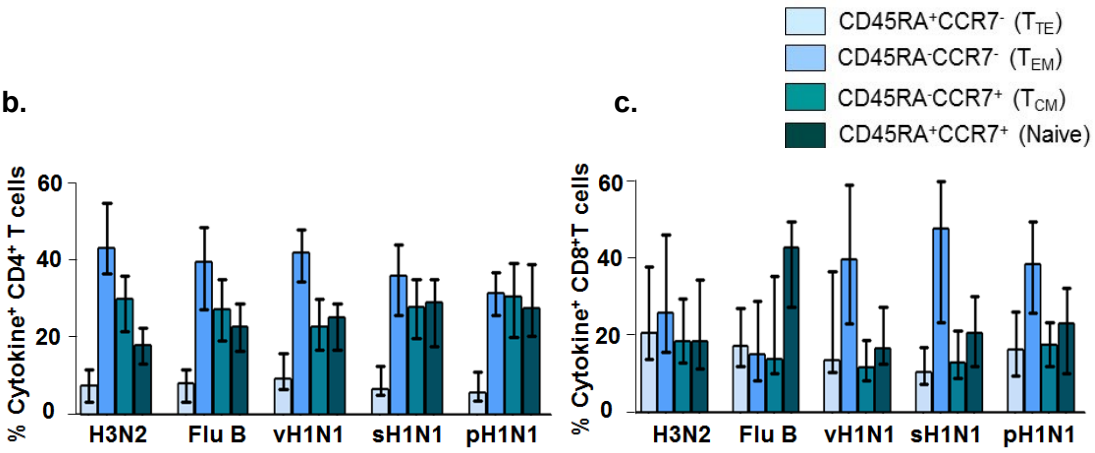


Fig 3.6. Total cytokine+ CD4$^+$ and CD8$^+$ T cells were categorized into various phenotypic subsets based on the surface expression of CD45RA and CCR7. (**a**) Gates were set on total CD4$^+$ and CD8$^+$ T cells and then applied to cytokine+ cells. To determine the phenotype of cytokine producing cells, we included only those samples with > 30 cytokine+ events and cytokine+ frequencies >3x over background (**b** and **c**). Phenotypes of cytokine+ CD4$^+$ (**b**) and CD8$^+$ (**c**) T cells after ex vivo stimulation with various influenza viruses as indicated. TIV and LAIV groups were combined at baseline (N=42).

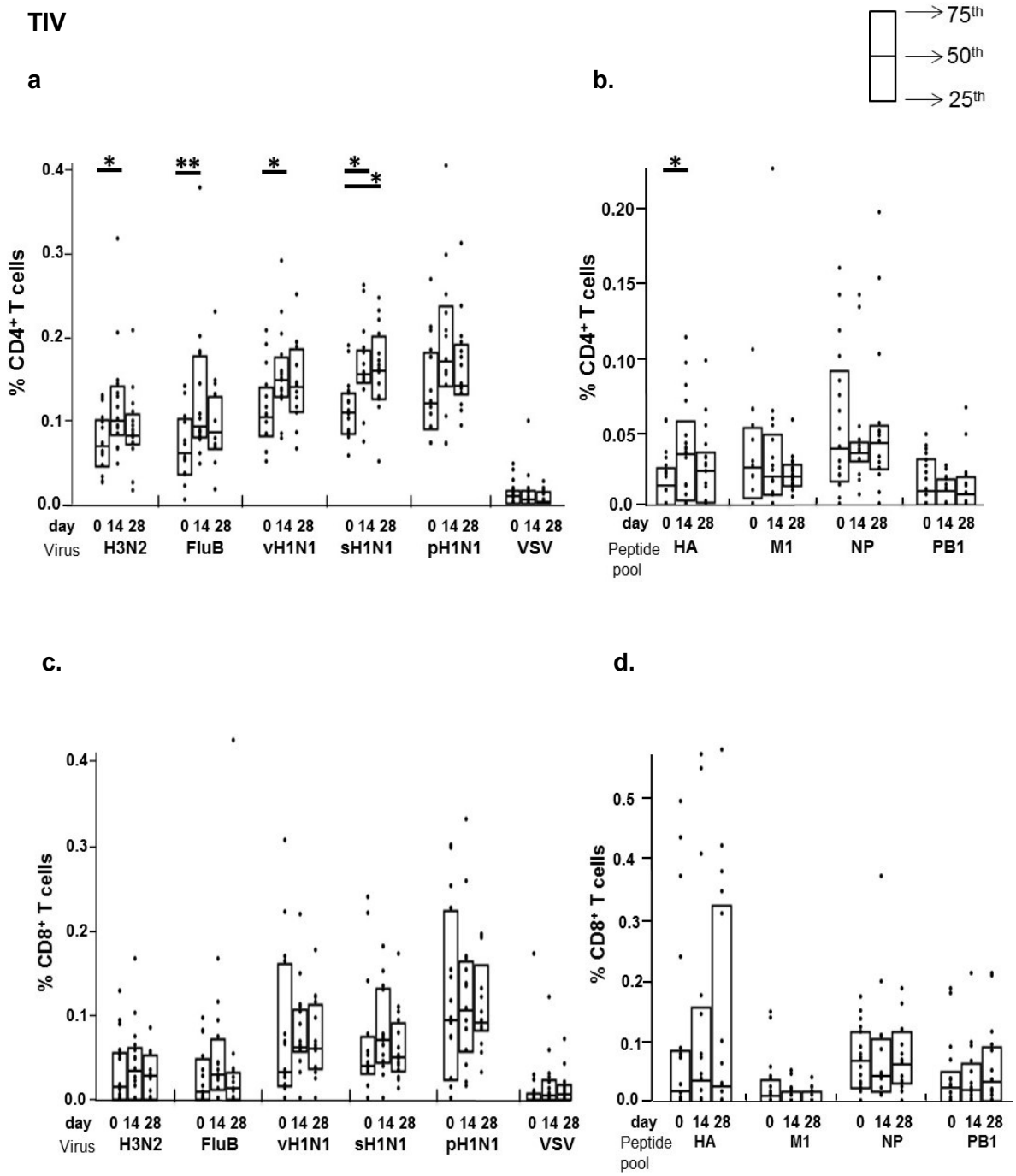
3.4.5 Effect of seasonal vaccination on influenza-specific CD4 and CD8 T cell frequency

We next examined, the effect of different seasonal vaccine delivery/formulation strategies on the magnitude and quality of CD4 and CD8 T cell responses. Fourteen days after intramuscular vaccination with TIV, we detected a significant increase in total cytokine producing CD4 T cells against influenza strains contained in the vaccine (**Fig 3.7a**). Interestingly, we also observed a significant increase in the frequency of influenza-specific CD4 T cells against antigenic variants not included in the vaccine including sH1N1 and importantly the pandemic 2009 H1N1 (pH1N1) virus, although the latter did not reach statistical significance (**Fig 3.7a**). By 28 days post vaccination, CD4 T cell frequencies against all strains, except sH1N1, had returned to levels similar to those prior to vaccination. The CD4 T cell response noted above was primarily directed against hemagglutinin (HA) as we observed a significant increase in CD4 T cells against HA

(derived from hemagglutinin of A/Wisconsin/67/2005 (H3N2)) but not against any of the internal proteins tested at day 14 post vaccination with TIV (**Fig 3.7b**). These data are consistent with the fact that the inactivated vaccine used in this study (Fluzone, Aventis Pasteur), is a split vaccine (www.fda.gov/downloads/BiologicsBloodVaccines/ApprovedProducts/UCM305089.pdf) predominantly composed of hemagglutinin (HA) with internal proteins present at much lower concentrations (234). In contrast to the CD4 T cell response to TIV, we did not observe any changes in virus or protein-specific CD8 T cells after vaccination (**Fig 3.7c** and **d**).

We and others have previously reported weak antibody responses to LAIV in adult vaccine recipients (162,235). It has therefore been suggested that T cell immunity may contribute to protection following LAIV (85). Indeed, we found that vaccination with LAIV induced a significant increase in total cytokine producing CD4 T cells against vH1N1 (detected at 28 days post vaccination, $p<0.05$) and Flu B (detected at 14 and 28 days post vaccination, $p<0.05$) (**Fig 3.7e)**. CD4 T cell frequencies against sH1N1 and pH1N1 also showed a trend towards an increase at day 28 post-vaccination, although this did not reach statistical significance. When we examined the protein-specificity of the CD4 T cell response to LAIV, we did not observe any significant changes in CD4 T cell frequencies against any of the tested proteins (**Fig 3.7f**). In addition, we did not observe any changes in the frequency of total cytokine producing CD8 T cells against influenza viruses (**Fig 3.7g**) or proteins (**Fig 3.7h**) following vaccination with LAIV.

Figure 3.7 Effect of seasonal vaccination on influenza-specific CD4 and CD8 T cell frequency



LAIV

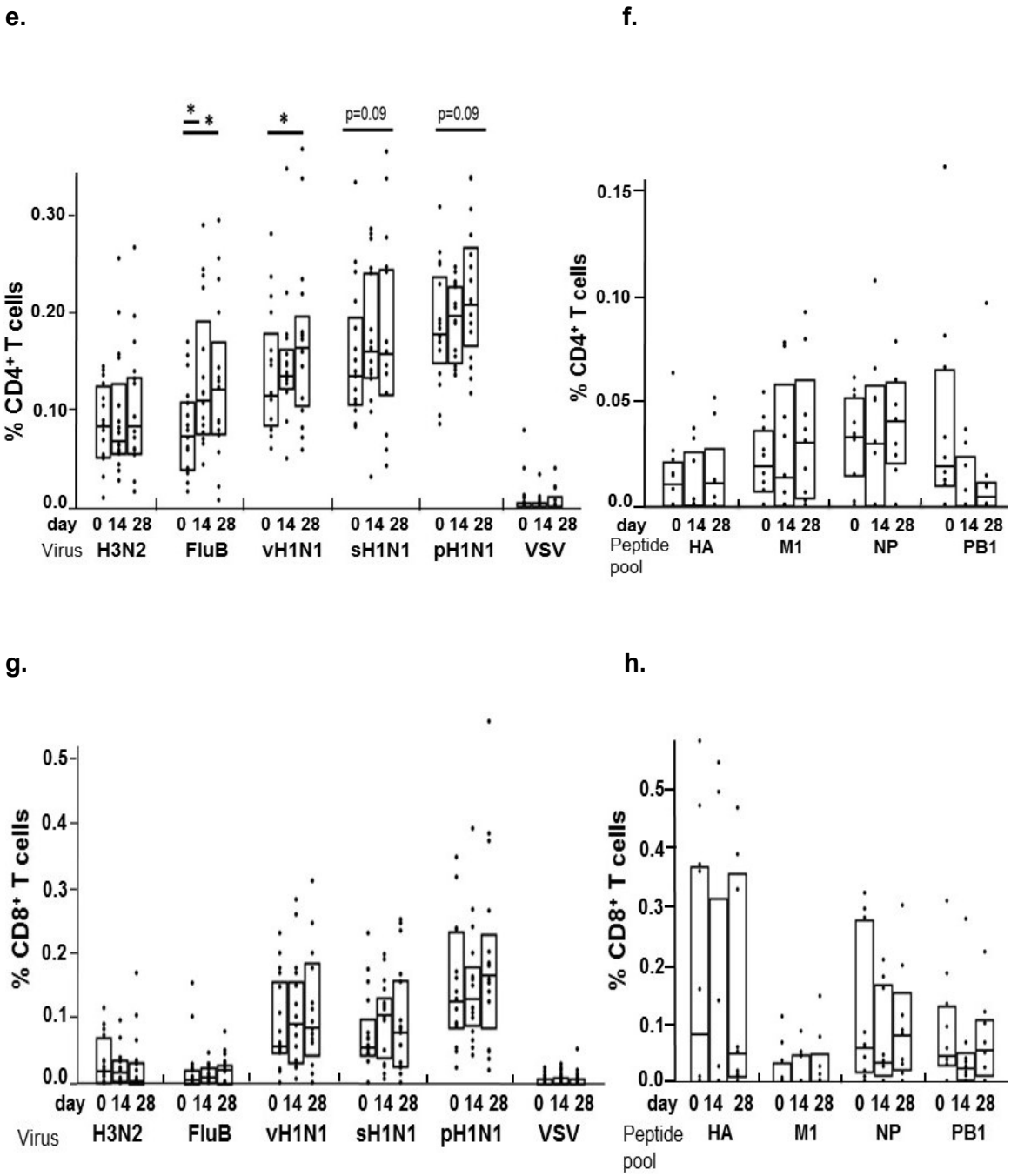


Fig 3.7. PBMCs were stimulated *ex vivo* with whole, β-propiolactone inactivated influenza viruses or peptide pools as indicated. The frequency of total cytokine positive (IFN-γ, TNF-α, IL-2 or any combination of the three cytokines) CD4 and CD8 T cells was enumerated at day 0 (baseline), day 14 and day 28 post-vaccination with TIV (n=25) (**a-d**) and LAIV (n=17) (**e-h**). Individual values are represented as dots. *p<0.05, **p<0.01 Wilcoxon signed rank test.

3.4.6 Effect of seasonal vaccination on influenza-specific T cell quality

To determine if changes in the frequency of total cytokine producing cells was accompanied by changes in T cell quality, we next investigated the cytokine secretion profiles of CD4 and CD8 T cells at various time points after vaccination with TIV and LAIV. Interestingly, vaccination with TIV was associated with a reduction in the proportion of single cytokine producing cells in favor of double producers at day 14 and day 28 post vaccination (**Fig 3.8**). These changes were however not uniform and could only be detected against H3N2, Flu B (both present in seasonal TIV) and interestingly, against pH1N1 as well. In contrast, no significant changes in cytokine secretion profiles were noted in CD8 T cells or in LAIV recipients (data not shown).

Figure 3.8 Effect of TIV on T cell quality

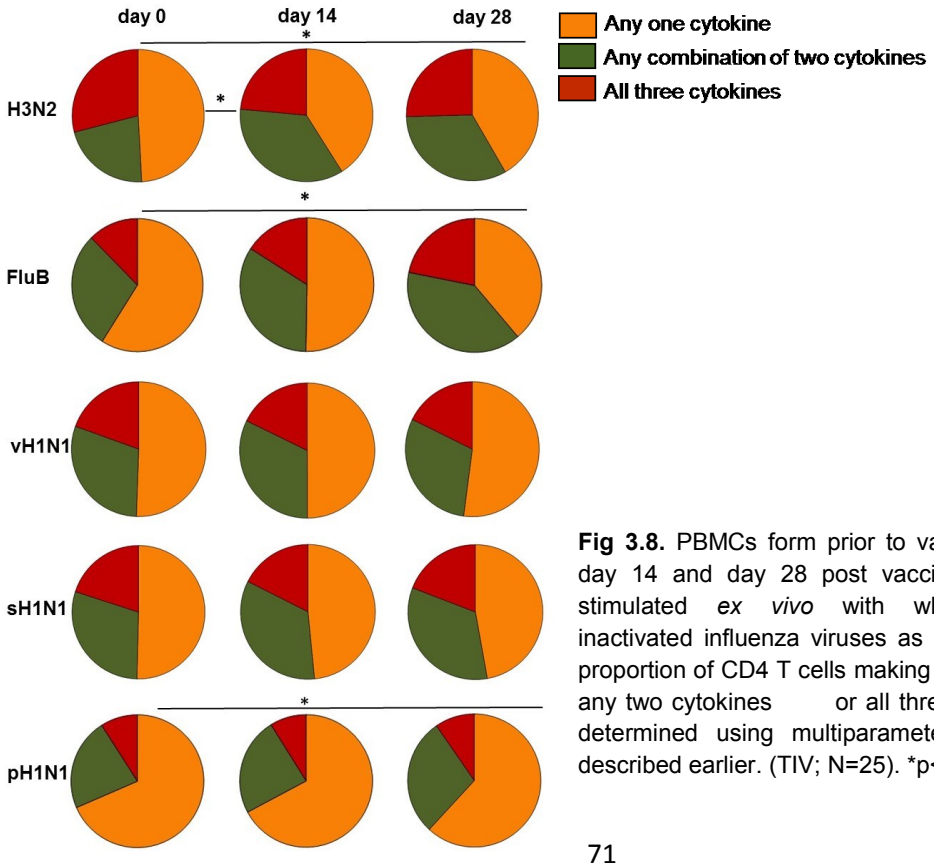


Fig 3.8. PBMCs form prior to vaccination (day 0) and day 14 and day 28 post vaccination with TIV were stimulated *ex vivo* with whole, β-propiolactone inactivated influenza viruses as indicated. The relative proportion of CD4 T cells making any one cytokine , any two cytokines or all three cytokines was determined using multiparameter flow cytometry as described earlier. (TIV; N=25). *p<0.05

3.5 Discussion

Despite vaccination efforts, influenza remains a major cause of morbidity and mortality worldwide (2,236). The current vaccines show limited efficacy in vulnerable populations such as the elderly and in instances when there is poor antigenic match between vaccine and circulating strains (123). These limitations represent an important challenge for new vaccine efforts, particularly in light of the constant threat posed by pandemic strains which can be highly virulent and pathogenic (237,238).

Current FDA guidelines for the assessment of immunological endpoints of vaccine effectiveness are restricted to antibody-based benchmarks (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074786.htm and http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074794.htm). Studies in animal models and more recently in humans, point to an increasingly important role for cellular immune responses in mediating cross-reactive or heterosubtypic immunity to influenza (78,81). In addition, induction of T helper cells following vaccination has been found to correlate with an increase in antibody titers in humans lending support to the notion that optimum antibody production requires concomitant T cell activation (68,79,239,240). Harnessing cellular immune responses to enhance antibody production as well as offer broad protection against a range of circulating and emergent pandemic strains would be of great benefit from a vaccination standpoint. Nevertheless, very little is known about influenza-specific T cell immunity or vaccination strategies that can induce such cellular responses, particularly in humans. Accordingly, in the present study we examined the cellular immune response to influenza. In addition, we used the commercially available seasonal

influenza vaccines as a tool to understand how different immunization routes and antigen formulations affect cell mediated immunity against influenza.

Early studies examining the pathogenicity of the 2009 pandemic H1N1 virus (pH1N1) using animal models suggested increased virulence compared to seasonal influenza viruses (241-243). However, in humans, overall morbidity and mortality associated with pandemic H1N1 infections was low and comparable to seasonal influenza infections despite the poor cross-reactivity of antibodies to pH1N1 in most young adults (244-246). In our cohort, we were able to detect a high degree of cross-reactivity in influenza specific memory T cells compared to antibodies against drifted strains as well as pH1N1 prior to vaccination. These observations are in line with previous studies that report a higher conservation of T cell epitopes compared to B cell epitopes between circulating seasonal H1N1 strains and pH1N1 (75). The presence of such broadly cross-reactive T cells may explain why most people did not become severely ill despite the low levels of pre-existing antibodies against pH1N1. This assertion is supported by previous studies demonstrating a key role for pre-existing T cells in mediating protection against severe disease in humans (78,81). Interestingly, *Wrammert, et al.* report the presence of rare populations of memory B cells that are broadly cross-reactive in nature, despite the absence of cross-reactive antibodies. They propose a model wherein expansion of these rare memory B cells following infection mediates protection from newly emerged influenza strains (87). The relative contribution of these different cell types to protective immunity is still not clear and warrants further investigation.

More than a decade ago it was proposed that circulating memory T cells in humans could be divided into two distinct subsets on the basis of proliferative capacity and migration potential: central memory (T_{CM}) cells that express CCR7 and CD62L (L-selectin) and proliferate extensively and effector memory (T_{EM}) cells that do not express

these markers and are less proliferative but capable of rapid effector function (233). While factors that favor the generation of one population over the other have not been fully identified, acute infections are thought to favor the generation of T_{CM} whereas chronic infections as well as repeated antigenic stimulation are thought to progressively skew towards a T_{EM} phenotype with a substantially reduced proliferative and IL-2 producing capacity (247,248). Previous studies examining the phenotype of influenza-specific T cells are divided: some have noted a preponderance of central memory T cells while others have reported primarily an effector memory phenotype (75,249). Our data suggest that these differences may in part be due to difference in the viral strains tested although the mechanisms that drive this differential response are still not clear. In general, we found that the majority of influenza-specific CD4 and CD8 T cells expressed a T_{EM} phenotype (Fig 3.6b and c) and CD8 T cells in particular had a limited capacity to produce IL-2 (data not shown) consistent with the notion that repeated seasonal infections favors the generation of memory cells with an effector memory phenotype. Interestingly, as shown previously (250), we too found a sizeable proportion of cytokine+ T cells to have a naïve-like phenotype. It is tempting to speculate that these naïve-like memory cells may belong to a recently identified population of memory "stem cells" which have a high capacity for self-renewal and an ability to derive T_{CM}, T_{EM} and effector populations (251). An important unanswered question is the relative contribution of these memory subsets to protective immunity as well as the lineage relationship between them. On the one hand induction of T_{EM} within non-lymphoid tissues may be desirable for vaccines against pathogens that replicate in peripheral tissues such as influenza (252-254). In support of this, a recent study showed that the presence of influenza-specific memory CD8 T cells that expressed a T_{EM} phenotype correlated with protection from severe disease (81). On the other hand, induction of self-renewing, proliferative T_{CM}

or stem cell-like memory populations may confer long-term protection against infection (255-257). More studies are required to dissect the relative contribution of different memory CD4 and CD8 T cell subsets to protective immunity to influenza.

The functionality of antigen-specific memory T cells has also recently emerged as an important predictor of protection (215). Reports from a number of different disease models in mice, non-human primates and humans suggest that memory CD4 and CD8 T cells that express multiple effector functions, such as concomitant production of IFN-γ, TNF-α and IL-2, constitute a higher quality response than their single-positive counterparts (59,189,249,258,259). Immunological mechanisms that drive the generation of specific functional profiles are poorly defined and represent a major hurdle in the rational design of new vaccines. One intriguing finding in this study was the reduced multifunctional potential of cross-reactive memory CD4 and CD8 T cells against pH1N1 compared to seasonal H1N1 viruses. While our data clearly demonstrated that memory CD4 and CD8 T cells established by seasonal infections and/or vaccinations recognize conserved epitopes in novel influenza viruses, some studies suggest that as many as half the conserved epitopes have at least one amino acid substitution thereby affecting the avidity of binding (260,261). Recent work in mouse models suggest a correlation between T cell functionality and avidity such that quality of the CD8 T cell response is enhanced in highly avid CD8 T cells (262). The reduced polyfunctionality of cross-reactive CD4 and CD8 T cells noted in this study may therefore reflect reduced functional avidity and warrants further investigation.

Another striking finding in this study was that while TIV and LAIV significantly enhanced the overall magnitude of the influenza-specific CD4 T cell response, only TIV induced a modest, yet significant, expansion of double cytokine producing cells. These data lend support to the idea that different vaccine types can differentially influence on

the multifunctional potential of the ensuing memory response (215). This finding is in keeping with studies in animal models that similarly noted a significant increase in double cytokine producing cells after subunit vaccination but not after administration of a live vaccine (230).

Several independent lines of evidence indicate that the successful vaccination strategies against pathogens such as malaria, tuberculosis, HIV and influenza will likely require the induction of robust cellular immune responses (206,263). This study as well as other demonstrate that in humans, pathogen-specific T cell responses are functionally and phenotypically heterogeneous and that simultaneous evaluation of T cell phenotype and function can provide a more sensitive and specific surrogate than T cell frequency alone. Flow cytometry is arguably the most advanced technology currently available for the immunophenotyping of T cells (264). However, multiparameter flow cytometry poses several technical and analytical problems including variability in reagents, sample handling and instrument settings. The use of pre-configured lyophilized reagents "lyoplates" has previously been shown to reduce variability in a multi-site study (224,225). In this study we extended this assay to include whole viral stimulations in addition to previously tested peptide pool-based approaches. Peptide based approaches alone are limited by the number of epitopes that can be examined and the use of overlapping peptides that cover the entire genome of the virus is expensive and may be impractical in larger trials. Stimulation with whole virus preparations on the other hand can enhance the sensitivity of detection of virus specific T cells against previously defined and undefined epitopes in a cost effective manner. In this study, we were able to detect robust T cell responses to whole inactivated viruses as well as peptide pools. Interestingly, despite using inactivated influenza viruses for stimulation of T cells, we were able to detect robust CD8 T cell responses suggesting effective cross-presentation

of exogenous antigens as reported elsewhere (265). Data analysis represents another major bottleneck and source of variability in multiparameter flow cytometry assays (266). Advances in automated gating strategies (267) as well as the development of newer software that allow for high-dimensional data analysis (268) represent crucial next steps in the standardization of flow cytometry assays and increasing their ease of use.

One of the goals of this study was to compare the impact of two different vaccination platforms on the development of antigen-specific T cell responses. Due to practical considerations, this study only examined peripheral T cells responses and not those in in local tissue, namely the lung. This sampling bias is particularly relevant when considering the immune response to a mucosally delivered vaccine such as LAIV. Indeed, several studies have noted the presence of a stable population of influenza-specific tissue resident memory T cells (T_{RM}) in the lungs of mice and humans that are distinct from circulating memory T cell subsets described earlier (269,270). It has been suggested that these highly localized memory T cells may act as a first line of defense against influenza (271) and may be more greatly impacted by mucosal vaccines such as LAIV than the peripheral responses measured in this study. Therefore caution must be used in interpreting our finding of stronger CD4 T cell responses following vaccination with TIV compared to LAIV. Future studies in which T_{RM} populations are examined through direct bronchial sampling may provide more insight into the immunological underpinning of LAIV.

To our knowledge, this study represents the most comprehensive examination of influenza-specific T cell immunity in humans. We have made several key observations regarding the heterogeneity of influenza-specific T cells as well as demonstrated a differential impact of the two seasonal vaccines on the magnitude and quality of this response. Future studies are required to determine if the vaccine induced improvement

in T cell quality or cross-reactivity observed in this study will serve to limit disease severity in a clinical setting.

CHAPTER 4: Role of IL-10 in the pathogenesis of influenza

4.1 Abstract

We examined the impact of IL-10 deficiency on disease outcome following lethal infection with influenza. We found that IL-10 deficient mice had significantly higher survival rate compared to WT mice suggesting a pathogenic role for IL-10. The higher survival in *Il10*$^{-/-}$ correlated with improved lung function and significantly reduced pulmonary injury. Surprisingly, despite increased expression of several proinflammatory cytokines including TNF-α, IL-6 and IFN-γ, *Il10*$^{-/-}$ mice did not have lower pulmonary viral load. However, correlating with improved survival, lungs of *Il10*$^{-/-}$ mice had significantly higher levels of IL-22 and IL-22 expressing CD4 T cells. IL-22 is a cytokine that has previously been shown to be important in mediating recovery from influenza infection. Interestingly, protection in *Il10*$^{-/-}$ was found to be independent of IL-22 as *Il10*$^{-/-}$*Il22*$^{-/-}$ mice had survival rates similar to that of *Il10*$^{-/-}$ mice. Finally, we show that impact of IL-10 on disease susceptibility may be influenza strain-specific.

4.2 Introduction

Seasonal influenza infections cause substantial morbidity and mortality resulting in an estimated 250,000-500,000 deaths worldwide every year. Young children and adults over the age of 65 as well as those with chronic medical conditions greatly increases the risk of developing severe complications with seasonal influenza (272,273). Despite having a lower risk of complications, around 6000 to 7000 young, healthy individuals between 18-64 years of age succumb to seasonal infections every year in the United States. During influenza pandemics, this number is typically much higher. During the 1918 pandemic, for instance, it was estimated that individuals between the ages of 20-40 accounted for approximately half of the total influenza deaths (274). Similarly, in 2009, young adults were at highest risk for influenza-related morbidity and mortality (275).

Pneumonia and the acute respiratory distress syndrome (ARDS) account for majority of the influenza-related complications during severe seasonal and pandemic infections and is most commonly categorized as primary viral pneumonia or a secondary bacterial pneumonia (276). Primary viral pneumonia occurs when the viral infection extends distally to the lung resulting in severe acute alveolar injury (also called diffuse alveolar damage) characterized by the formation of hyaline membranes lining the alveoli, necrosis of alveolar epithelium, flooding of alveolar lumen by edema fluid and substantial intra-alveolar hemorrhage in addition to necrotizing bronchitis and bronchiolitis (113,276). This pulmonary injury is accompanied by an elevated macrophages and lymphocytes in pulmonary infiltrates as well as a significant increase in pro-inflammatory cytokines and chemokines in serum (99,277-280). Findings in mice and non-human primate models of infection closely mirror that of humans (281). The significant increases in cytokine and chemokines and the corresponding immune dysregulation, otherwise

called the “cytokine storm”, is thought to, in part, underlie the observed pathology during primary viral pneumonia. Indeed, pharmacological interventions which blunt overall levels of pulmonary inflammation can significantly enhance survival in animal models, lending further support to this hypothesis (282). Similarly, reducing inflammatory cell recruitment, particularly innate immune cells, into pulmonary tissue has also been shown to markedly improve survival in mice (96).

IL-10 is a potent anti-inflammatory cytokine with a key role in limiting inflammatory and autoimmune pathologies (283). The importance of IL-10 in controlling inflammation is evident in numerous models including *Toxoplasma*, *Listeria*, *Plasmodium*, experimental autoimmune encephalomyelitis (EAE) and models of asthma, where *Il10*$^{-/-}$ mice develop severe pathological lesions due to excess inflammation (284-289). The substantial immunopathology observed in *Il10*$^{-/-}$ suggest that the absence of IL-10 cannot be compensated by other regulatory mechanisms and that it plays a non-redundant and crucial role in controlling inflammation *in vivo.* Somewhat paradoxically, IL-10 has also been associated with detrimental disease outcomes due to its ability to suppress the development of an effective immune response. For instance, during infection with *Leishmania*, elevated IL-10 has been linked with increased parasite burden in animal models and humans (290-292). Similarly, IL-10 has also been shown to negatively affect clearance of *M. tuberculosis* and lymphocytic choriomeningitis virus (LCMV) in animal models (293,294)

Interestingly, while IL-10 production has been described following influenza infection in mice and humans, its role in influenza pathogenesis is not clear (295-298). In humans, high IL-10 levels are associated with negative outcomes in hospitalized individuals suggesting that IL-10 expression during influenza infection may be detrimental to the host (107-109). However, studies in animal models have yielded conflicting results with

one study suggesting that IL-10 expression is critical in controlling pulmonary inflammation and injury (110) while other studies suggest that IL-10 is pathogenic during influenza infections (112,299).

In this study, we utilized *Il10*$^{-/-}$ mice to characterize the role of IL-10 in inflammatory cell recruitment, development of pulmonary pathology and ultimately respiratory dysfunction during lethal influenza infections *in vivo*. The data presented in this chapter suggest that IL-10 contributes to influenza induced pulmonary pathology and vascular dysfunction without significantly affecting viral clearance. Future studies aimed at elucidating the effect of IL-10 on vascular permeability may provide more insight into mechanisms underlying the observed findings.

4.3 Materials and Methods

Mice

Female WT, *Il10*$^{-/-}$, *Il22*$^{-/-}$ or *Il10*$^{-/-}$x*Il22*$^{-/-}$ were between 9-12 weeks old at the time of virus infection. All lines were on the C57Bl/6 background. WT mice were obtained from Jackson Laboratory whereas, *Il10*$^{-/-}$ were bred in the animal breeding facility at Johns Hopkins. WT and *Il10*$^{-/-}$ mice were negative for helicobacter. *Il22*$^{-/-}$ mice were kindly provided by Dr. Ouyang from Genentech and were backcrossed to *Il10*$^{-/-}$ to generate *Il10*$^{-/-}$x*Il22*$^{-/-}$ mice. Experimental animal procedures were conducted in accordance with the Johns Hopkins Animal Care and Use Committee guidelines.

Virus stocks, titers and infections

Egg-grown, mouse-adapted influenza A virus, A/Puerto Rico/8/34 (A/PR8) was generously provided by Dr. Maryna Eichelberger at the U.S. Food and Drug Administration. A/WSN/33 was a kind gift from Dr. Andrew Pekosz at the Johns Hopkins School of Public Health. Virus stock titers or lung viral loads were determined by endpoint dilution assay and expressed as tissue culture infectious dose 50 ($TCID_{50}$) as described elsewhere (110). Briefly, Madin-Darby canine kidney (MDCK) cells were incubated with 10-fold serial dilutions of viral stocks or whole lung homogenates from influenza infected mice for 3 days at 37ºC in a humidified atmosphere of 5% CO_2. After 3 days, supernatants were collected and mixed with half volume 0.5% chicken red blood cells (CBT farms, Federalsburg, MD), the agglutination pattern read and $TCID_{50}$ values calculated using the method of Reed and Muench.

For *in vivo* infections, mice were anesthetized with intramuscular injection of Ketamine/Xylazine (80 mg/Kg and 6 mg/Kg, respectively) and infected intranasally with

30 µl of Phosphate Buffered Saline (PBS) containing A/PR/8 or A/WSN/33 at doses indicated in the results.

Real-time PCR for viral load and cytokine gene expression

At different time points after intranasal infection, mice were euthanized and lungs were perfused by injecting 20 ml of PBS into the left ventricle of the heart. For cytokine gene expression studies and viral load determination, the left lung was placed in TRIzol (Cat. No: 15596-018, Life Technologies). RNA was isolated from whole lung homogenates using TRIzol and reverse transcribed using first-strand cDNA synthesis kit (Cat No. 18080-051, Invitrogen), according to manufacturer's protocol. Quantitative PCR was performed using SYBR Green primers for cytokine genes (Appendix: Table 1). For viral load quantification, the polymerase (PA) gene of A/PR8 was amplified using Taqman primers and probes as described elsewhere (299). The fold increases in relative signal to that of uninfected samples was determined by the ΔΔCT calculation as recommended by Applied Biosystems. All analyses were performed on an ABI 7300 Real-Time PCR System.

Cytokine analyses in bronchoalveolar lavage fluid (BALF)

Mice were killed at the indicated time points by an overdose of ketamine and xylazine and were subjected to bronchoalveolar lavage (BAL) 3 times with a total of 1 ml of phosphate buffered saline (PBS) instilled via the trachea. BAL fluid (BALF) was centrifuged at 2000 rpm for 5 minutes at 4ºC and the supernatant was used for subsequent measurement of cytokines. Levels of IL-6, TNF-α, CCL2, IFN-γ, IL-12p70 and IL-10 in BALF was determined using mouse proinflammatory BD CBA kit (Cat No.

552364, BD Biosciences). Levels of IL-22 was determined using FlowCytomix (Cat No. BMS86022FF, eBiosciences). Levels of IL-17 were determined using FlowCytomix (Cat No.BMS86001FF, eBiosciences) and BD Flex Set (Cat No.560283, BD Biosciences).

Pulmonary function analysis

Animals were anesthetized with Ketamine and Xylazine as described above and cannulated with an 18-guage stub needle cannula via the trachea. Measurement of DF_{CO} was performed as mentioned elsewhere (300). Briefly, 0.8 ml of a gas mixture of approximately 0.5% neon, 0.5% CO and balance air was used to inflate the lung via the cannula. After waiting 9 seconds, 0.8 ml was withdrawn from the lung and injected into a desktop Gas Chromatograph (Inficon Micro GC model 3000A). The DF_{CO} is defined as 1-(CO_9/CO_c)/ (Ne_9/Ne_c) where the subscripts c and 9 stand for the calibration gas injected and gas withdrawn after 9 seconds. Following DF_{CO} measurement, animals were artificially ventilated at a rate of 120 breaths/minute and a tidal volume of 0.2 ml. One minute after a 5 second deep inspiration, dynamic elastance was measured as described elsewhere (301).

Histopathological examination and TUNEL staining

For histopathological examination, lungs were inflated at a constant pressure of 30 cm H2O for 5 minutes with 10% neutral buffered formalin (NBF). Trachea were tied off and intact lungs were excised from the chest cavity and submerged in 10% NBF for at least 48 hours prior to paraffin embedding. Lungs were stained with hematoxylin and eosin (H&E) and blindly evaluated for pathological changes. For postmortem studies, lungs were collected within 5 hours of death. For day 8 post-infection, mouse lungs were collected after pulmonary function analysis.

For the detection of apoptotic cells, Tdt-mediated dUT-biotin nick-end labeling (TUNEL) staining was performed using the *in situ* cell death detection kit (Cat No.11 684 795 910, Roche) as per manufacturer's instructions. Slides were counter stained with DAPI to define nuclei. Rate of apoptosis was calculated by dividing the number of TUNEL positive cells by the total number of nuclei (DAPI+) in any given field of view. The prevalence of apoptosis for a given lung section was averaged over 5 randomly selected fields of view at 40x magnification. A total of 9 slides per group were analyzed over two independent experiments.

Flow cytometry and intracellular cytokine staining

For flow cytometric evaluation of pulmonary leukocyte populations, single cell suspensions were made from whole lungs by mechanical disruption using the Miltenyi gentleMACS cell dissociator (Cat No. 130-093-235) according to manufacturer's protocol. Single cell suspensions were stained with LIVE/DEAD Fixable Dead Cell Stain Kits (Cat No: L34959, Invitrogen) according the manufacturer's instructions followed by incubation with anti-CD16 Fc Block (Cat No:553142, BD Biosciences) for 20 minutes on ice to block FcRs. Cells were then stained with the following antibodies CD11b-PerCP-Cy5.5 (550993, BD Biosciences), CD11c-APC (550261, BD Biosciences), MHC II(I-A/I-E)-FITC (11-5321, eBiosciences), Ly6G-PE (551460, BD Biosciences), Ly6C-APC (17-5932-80, eBiosciences), CD4-APC-H7 (560181, BD Biosciences), CD8-Pacific Blue (558106, BD Biosciences), CD3-PE-Cy5 (15-0031-81, eBiosciences) and NK1.1-PE (553165, BD Biosciences) in wash buffer (1x PBS with 0.5% BSA) for 30 minutes on ice. Following surface stain, cells were washed twice in wash buffer.

For intracellular detection, single-cell suspensions of lungs were prepared as described above and restimulated for 5 hours with 40 ng/ml Phorbol-12-myristate-13-

acetate (PMA; Cat No:524400, Calbiochem) plus 2 ug/ml Ionomycin (Cat No: 407952, Calbiochem) at 37°C with brefeldin A (Cat No:51-2301KZ, BD Biosciences) added during the last 3 hours. Cells were washed, surface-stained as described above and then fixed and permeabilized by incubating in Cytofix/Cytoperm (BD Biosciences) for 20 minutes on ice. Cells were washed in Perm/Wash buffer (BD Biosciences) and immunostained with anti-IFN-γ-PE-Cy7 (557649, BD Biosciences), anti-IL-17-Alexa Fluor 488 (560220, BD Biosciences) and anti-IL-22-PE (12-7221-82, eBiosciences) in Perm/Wash buffer for 30 minutes on ice. FACS analysis was performed on a BD Bioscience LSR II and FlowJo (Tree Star) analysis software.

Statistical analysis

The log rank test was used to test for significant differences in Kaplan-Meier survival curves. Unpaired, two-tailed Student's *t* tests, $\alpha=0.05$, were used to determine if there were significant differences in the means of two normally distributed groups.

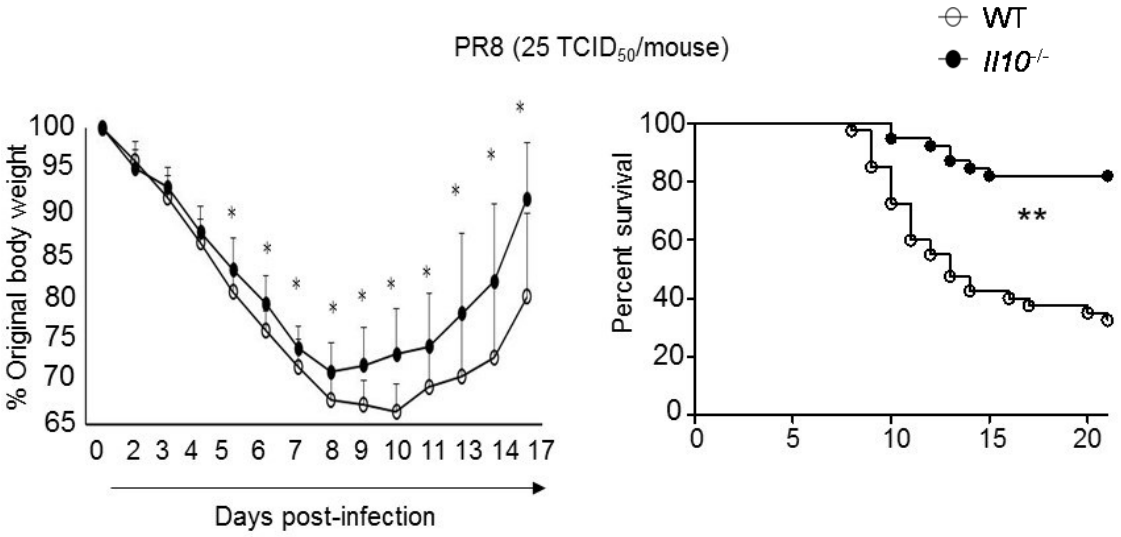
4.4 Results

4.4.1 IL-10 contributes to increased morbidity and mortality

To examine of the role of IL-10 in influenza pathogenesis, *Il10*$^{-/-}$ and WT mice were infected with different doses of influenza A/PR/8 and monitored daily for weight loss, as a measure of morbidity, and survival. As shown in **Fig 4.1a**, *Il10*$^{-/-}$ mice had significantly less weight loss than WT mice throughout the course of acute infection. This correlated well with higher survival rates, suggesting that IL-10 expression contributes to influenza disease severity. It should be noted that the weight loss was significantly higher for WT mice starting at day 5 post-infection until day 9 beyond which the data are subject to survival bias. To determine if this effect was dependent on the infectious dose, *Il10*$^{-/-}$ and WT mice were challenged with a higher dose (50 $TCID_{50}$/mouse) and a similar reduction in weight loss and mortality was observed in *Il10*$^{-/-}$ compared to WT mice (**Fig 4.1b**). Mice were infected with a dose of 25 $TCID_{50}$/mouse for the rest of the study.

Figure 4.1 Mortality and weght loss following infection with influenza A/PR/8

a



b.

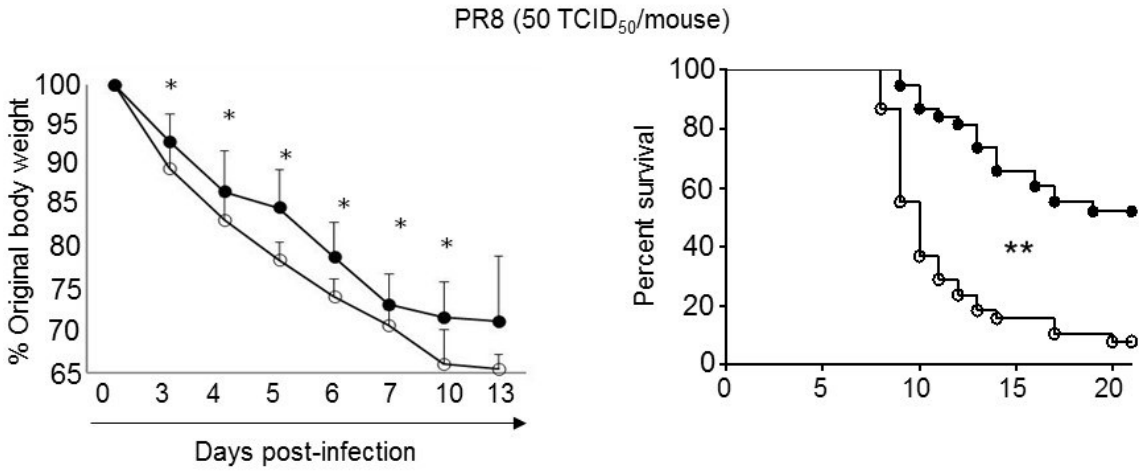


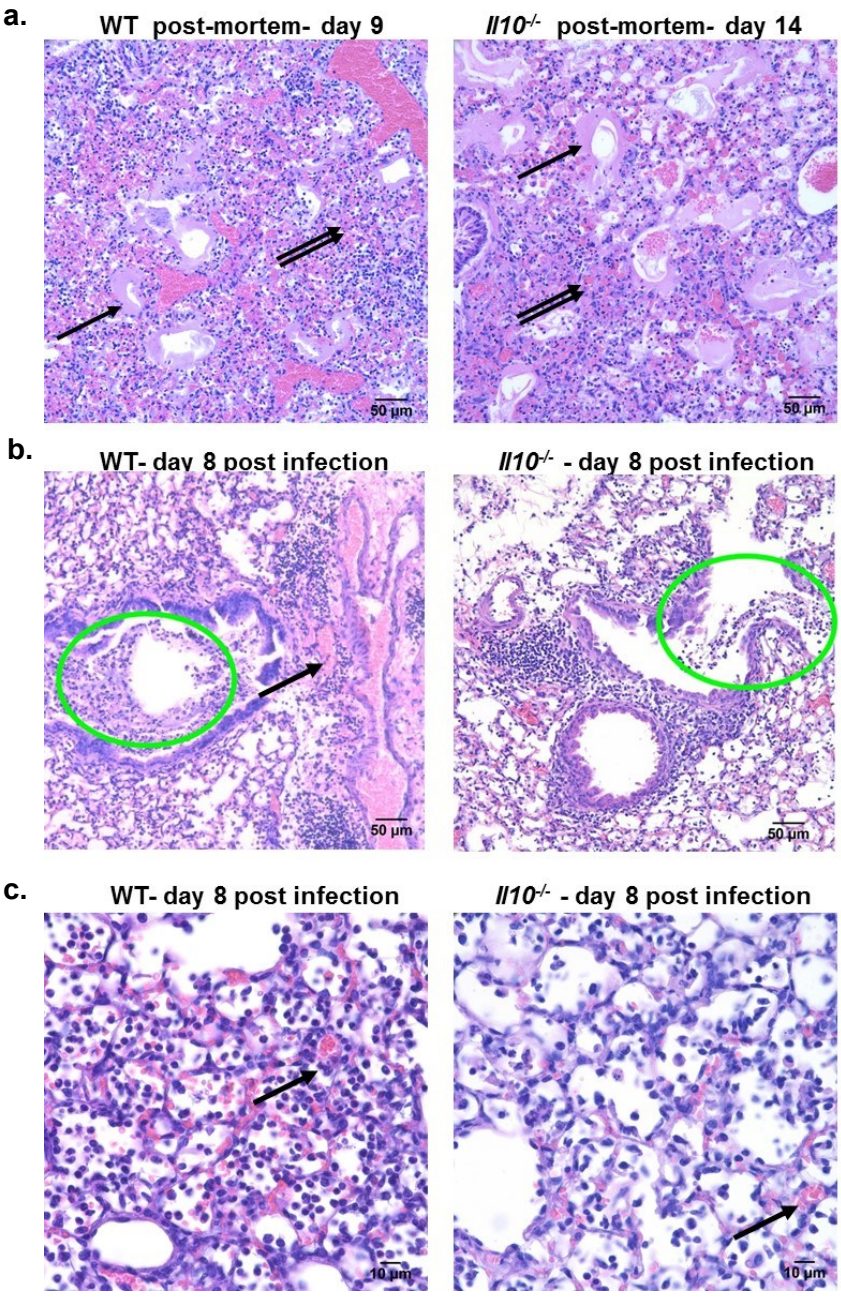
Fig 4.1. *Il10*$^{-/-}$ and WT were infected intranasally with (**a**) 25 $TCID_{50}$ or (**b**) 50 $TCID_{50}$A/PR/8. Weight loss and survival were assessed daily for a period of 21 days. (n=38-40 mice/group). Data were combined from 4 independent experiments, **p<0.01, log rank test, *p<0.01, student ttest)

4.4.2 Il10$^{-/-}$ *mice have decreased pulmonary pathology compared to WT*

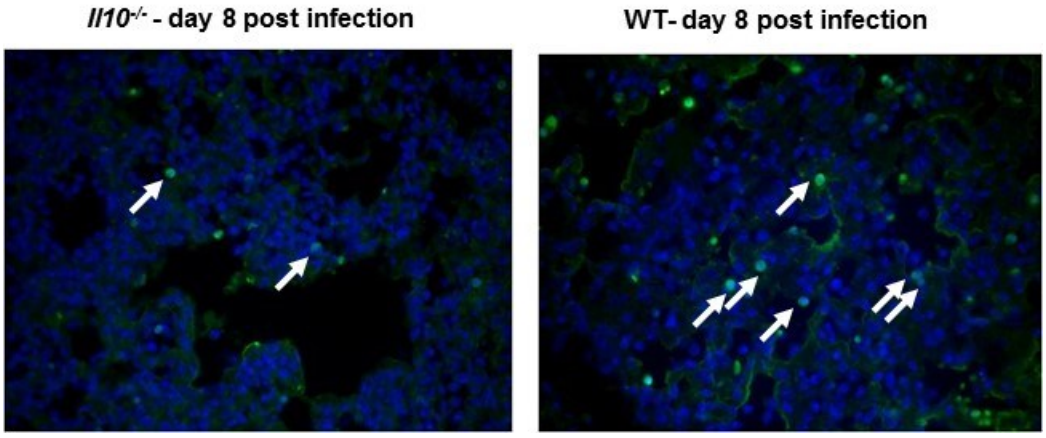
Next, to determine if IL-10 influenced pulmonary pathology, histological examination of lungs was performed postmortem in animals that had succumbed during the course of infection as well as at day 8 post-infection. Similar to findings in humans, postmortem examination of *Il10*$^{-/-}$ and WT lungs revealed pathological changes consistent with diffuse alveolar damage (DAD) characterized by the presence of hyaline membranes, infiltration of inflammatory cells and significant edema and hemorrhage (**Fig 4.2a**). Interestingly, while these pathological changes could be observed as early as day 9 post-infection in WT mice, similar changes became prominent only around day 14 in *Il10*$^{-/-}$ (data not shown). In mice that were euthanized at day 8 post-infection, histopathology confirmed patchy involvement of lungs and generally less airway involvement in *Il10*$^{-/-}$ compared to WT (**Fig 4.2b and c**). In addition, TUNEL staining at day 8 post-infection demonstrated a significantly lower number of apoptotic cells in the lungs of *Il10*$^{-/-}$

compared to WT mice (**Fig 4.2d and e**). Taken together, these data suggest that IL-10 deficiency results in a reduction and/or delay in pulmonary injury during acute infection leading to enhanced survival.

Figure 4.2 Histopathological changes in *Il10*$^{-/-}$ and WT lungs in PR/8 infected lungs



d.



e.

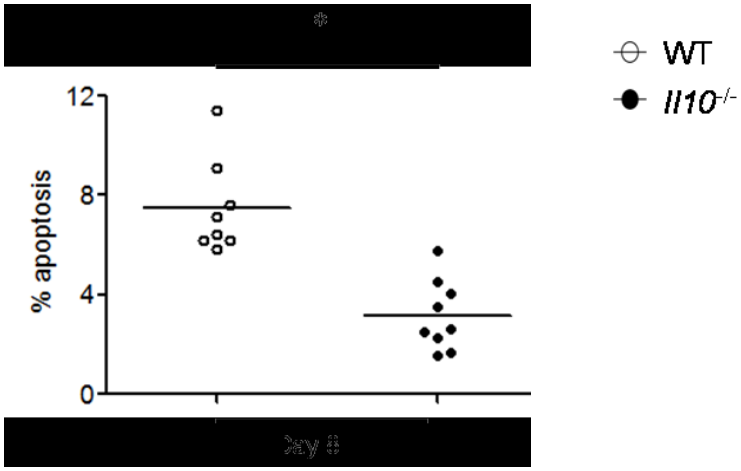


Figure 4.2. WT and *Il10*$^{-/-}$ mice were infected with A/PR/8 at a dose of 25 $TCID_{50}$. (**a**) Lungs were removed immediately post-mortem and fixed in 10% neutral buffered. H&E stained sections were examined blindly for pathology. Hyaline membrane (single arrow) and hemorrhage (double arrow) noted in *Il10*$^{-/-}$ and WT lungs (**b and c**) *Il10*$^{-/-}$ and WT mice were euthanized at day 8 post-infection and H&E stained lung sections were examined. (**b**) Presence of inflammatory exudate noted in *Il10*$^{-/-}$ and WT lungs (green circle). Perivascular hemorrhage (arrow) noted in WT lungs. (**c**) Alveolar inflammation and intraalveolar hemorrhage (arrow) more prominent in WT compared to *Il10*$^{-/-}$. (**d**) Induction of apoptosis was identified by *in situ* TUNEL staining of formalin fixed lung sections at day 8 post-infection. Slides were counter stained with DAPI (blue). Apoptotic cells are in stained in green (arrows) and nuclei are stained blue (**e**) The percentage of apoptotic cells per field of view was quantified as described in the methods (n=9 mice/ group from 2 independent experiments, *p<0.0001).

4.4.3 IL-10 does not affect viral replication

To determine if reduction in lung pathology was due to reduced viral replication in the lung, viral loads were determined at various time points after infection in whole lung homogenates by RT-PCR (**Fig 4.3**). Infectious viral load in whole lung homogenates were measured by end point dilution assay and were similar to RT-PCR results (data not shown). As shown in **Fig 4.3**, viral loads were similar in *Il10*$^{-/-}$ and WT mice throughout the course of infection suggesting that IL-10 does not play a role in controlling viral replication and that viral load does not account for histopathological difference in pulmonary injury between *Il10*$^{-/-}$ and WT mice.

Figure 4.3 IL-10 deficiency does not affect viral load

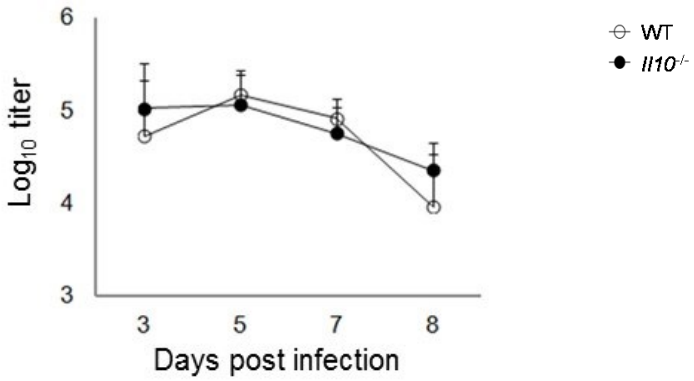


Fig 4.3. WT and Il10-/- were infected with 25TCID50/mouse of A/PR/8. Lungs were isolated at the indicated time points and viral load was quantified by semi-quantitative real-time PCR in whole lung homogenates at the indicated time points (n=8-10 mice/group/time point from 2 independent experiments)

4.4.4 Improved pulmonary mechanics in Il10$^{-/-}$ *mice compared to WT*

To determine if pathologic effects associated with influenza infection resulted in altered lung function, we assessed the diffusion factor for carbon monoxide-DF_{CO} (a metric that reflects the gas-exchanging ability of the mouse lung) and pulmonary

elastance during the course of infection (**Fig 4.4**). Consistent with histological findings in the lung during infection, changes in pulmonary mechanics were noted in both groups throughout the course of infection. Compared to WT mice, however, *Il10*$^{-/-}$ had significantly higher DF_{CO} values at day 6 and day 8 post-infection suggesting an enhanced capacity for gas exchange (**Fig 4.4a**). In addition, lungs from *Il10*$^{-/-}$ mice had significantly reduced lung elastance compared to WT mice at day 8 post-infection (**Fig 4.4b**). Taken together, these data suggest that in addition to reduced pulmonary pathology, *Il10*$^{-/-}$ mice also displayed significantly enhanced pulmonary function compared to WT mice.

Figure 4.4 *Il10*$^{-/-}$ have improved pulmonary function compared to WT

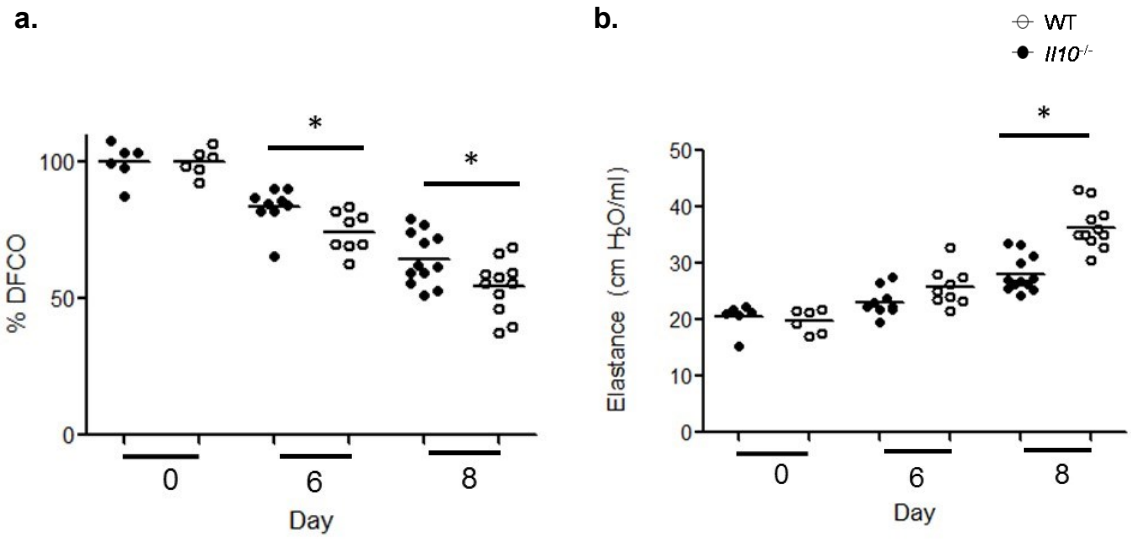


Fig 4.4. WT and *Il10*$^{-/-}$ mice were infected with A/PR/8 at a dose of 25 $TCID_{50}$. On the stated days (**a**) Diffusion factor of carbon monoxide (DF_{CO}) and (**b**) pulmonary elastance were measured (n=6-12 mice/ group/ time point, *p<0.05, **p<0.0001).

4.4.5 IL-10 deficiency does not substantially affect the recruitment of leukocyte populations, but results in an increase in proinflammatory cytokines

Because excessive inflammation is associated with severe influenza infections (95), we next examined the recruitment of inflammatory cell into the lungs of WT and *Il10*$^{-/-}$ mice (**Fig 4.5**). Interestingly, we detected no significant differences in any major infiltrating leukocyte populations including NK cells ($CD3^{-}Nk1.1^{+}$), neutrophils ($CD11b^{+}Ly6g^{+}Ly6c^{-}SSC^{hi}$) and dendritic cells ($CD11b^{+}CD11c^{hi}MHCII^{hi}$ and $CD11b^{-}CD11c^{hi}MHCII^{hi}$), at any time point post-infection. There was a significant increase in $CD4^{+}CD44^{+}$ T cells at day 3 and day 5 post-infection in WT mice compared to *Il10*$^{-/-}$, however, the numbers were similar by day 7 post-infection. No differences were observed in the recruitment of CD8 T cells between *Il10*$^{-/-}$ and WT mice.

The primary function of IL-10 is to negatively regulate the secretion of pro-inflammatory cytokines from leukocytes (283). Therefore, somewhat expectedly, the levels of numerous cytokines, including IFN-γ, TNF-α, IL-6 and IL-22 were elevated in the BALF of *Il10*$^{-/-}$ compared to WT (**Fig 4.6a**). Interestingly, no IL-17 could be detected at any time post infection in *Il10*$^{-/-}$ or WT BALF (data not shown). To rule out the possibility that the increased levels of cytokines in the BALF was due to increases in total protein leakage into the airways, total protein was also measured in BALF of *Il10*$^{-/-}$ and WT mice. However, no differences were observed in the levels of total protein between *Il10*$^{-/-}$ and WT (**Fig 4.6b**). In addition, consistent with elevated IL-22 and IFN-γ levels in the BALF, we also observed a significantly higher number of IL-22 and IFN-γ producing CD4 T cells in the lungs of *Il10*$^{-/-}$ compared to WT mice (**Fig 4.6c)**. No difference could be detected in the recruitment of IFN- γ producing CD8 T cells (data not

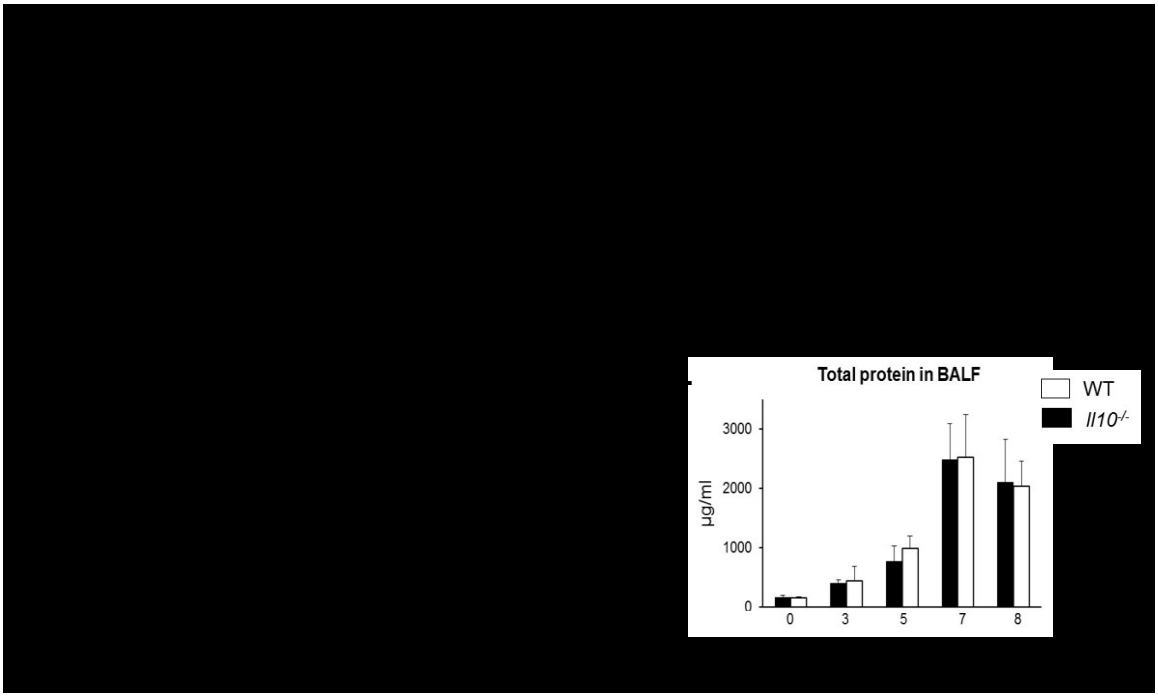
shown). No IL-22 producing CD8 T cells could be detected in *Il10*$^{-/-}$ or WT lungs (data not shown).

Figure 4.5 Recruitment of leukocytes following infection with influenza

Fig 4.5. WT and *Il10*$^{-/-}$ mice were infected with 25 $TCID_{50}$ of A/PR/8. Changes in the numbers of immune cells measured from whole lung by flow cytometry at days 0, 3, 5 and 7 post-infection. n=5-6 mice/ group/time point from 2 independent experiments. *p<0.05).

Figure 4.6 Expression of proinflammatory cytokines in the lungs of infected mice

a.



c.

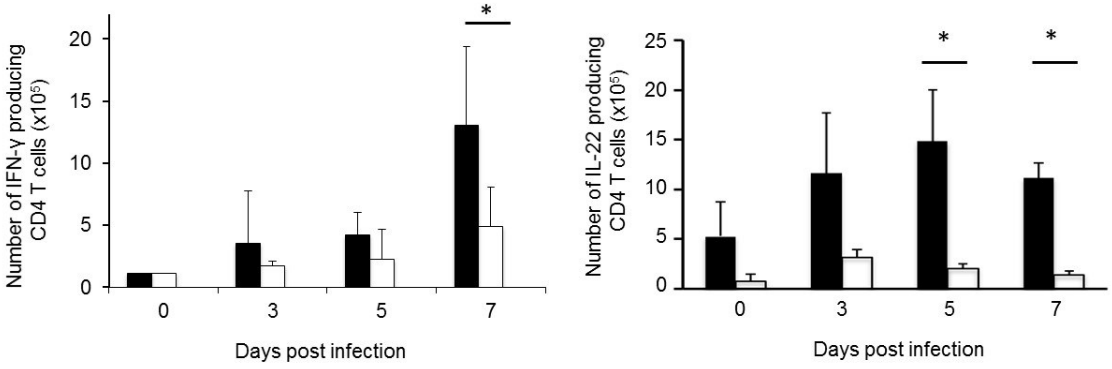


Fig 4.6 WT and *Il10*$^{-/-}$ mice were infected with 25 $TCID_{50}$ of A/PR/8. (**a** and **b**) At the indicated days post-infection, bronchoalveolar lavage fluid (BALF) was isolated and analyzed for protein levels of the stated cytokines (**a**) or total protein (**b**) (n=7-10 mice/ group/time point combined from 2 independent experiments. *p<0.05, **p<0.001). (**c**) Single cell suspensions of whole lungs were stimulated with PMA and Ionomycin in the presence of Brefeldin A and intracellular staining was performed for IL-22 and IFN-γ (n=3 mice/groups/time point from 2 independent experiments.*p<0.05)

4.4.6 IL-22 does not mediate enhanced survival to influenza challenge in Il10$^{-/-}$ mice

Previously published studies have identified an important role for IL-22 in mediating pulmonary repair following infection with influenza (302,303). Therefore, we explored the possibility that attenuated disease severity observed in *Il10*$^{-/-}$ mice was due to an up-regulation of IL-22 expression. To determine if there was a causal link between IL-22 and less disease in *Il10*$^{-/-}$, we generated *Il10*$^{-/-}$*Il22*$^{-/-}$ mice and then challenged them with a lethal dose of influenza A/PR/8 (25 $TCID_{50}$/mouse). Interestingly, despite numerous studies linking IL-22 expression with improved morbidity in WT mice, as shown in **Fig 4.7**, survival and weight loss in *Il10*$^{-/-}$*Il22*$^{-/-}$ mice was similar to that seen in *Il10*$^{-/-}$. These data suggest that increased production of IL-22 in *Il10*$^{-/-}$ mice does not affect disease outcomes.

Figure 4.7 IL-22 does not impact survival in Il10-/- mice

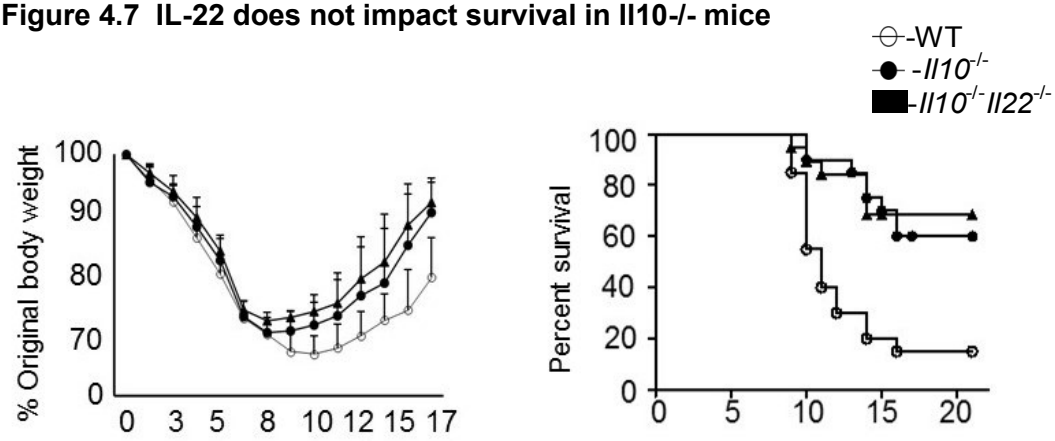


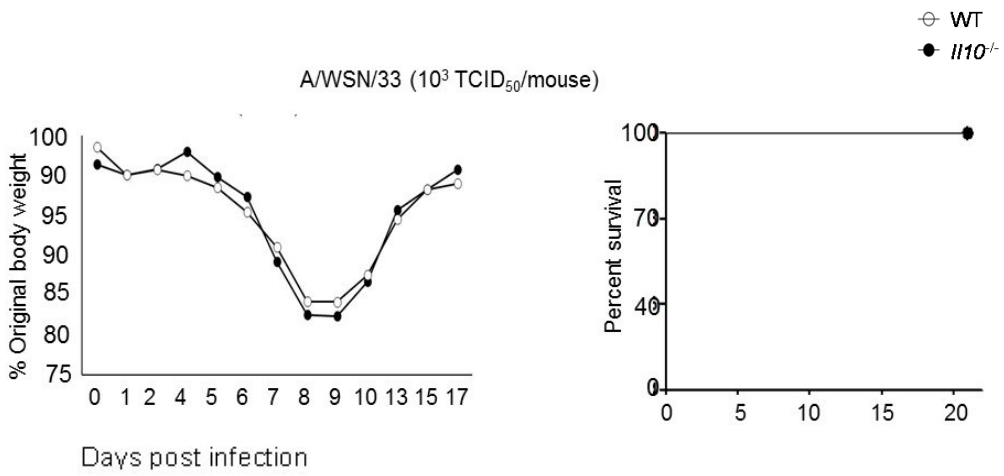
Fig 4.7. *Il10*$^{-/-}$*Il22*$^{-/-}$, *Il10*$^{-/-}$ and WT mice were infected with 25 $TCID_{50}$ of influenza A/PR/8 and monitored daily for survival and weight loss (n=20 mice/group from 2 independent experiments

4.4.7 The absence of IL-10 in A/WSN/33 infected mice does not affect disease outcome

Influenza viruses display a remarkable heterogeneity in terms of their ability to cause disease in humans. For instance, the highly pathogenic avian influenza strain, H5N1, is estimated to have a case fatality rate of approximately 60% compared to less than 0.1% with seasonal influenza viruses (www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/index.html.). Similarly commonly used mouse-adapted influenza viruses can also differ in their virulence with A/PR/8 representing a highly virulent strain and A/WSN/33 a less virulent one (304). Therefore, we next questioned whether IL-10 deficiency would also be beneficial during a less severe viral lung infection. To this end, we infected *Il10*$^{-/-}$ and WT mice with different doses of the less virulent A/WSN/33 strain and followed weight loss and survival. Surprisingly, we found that in contrast to the negative impact IL-10 had on disease outcome in A/PR/8 infected mice (**Fig 4.1**), it had no effect on disease progression in A/WSN/33 infected mice in terms of survival or weight loss (**Fig 4.8a** and **b**). To rule out the possibility that IL-10 may not be expressed during A/WSN/33 infection, IL-10 gene expression was measured by RT-PCR in whole lung homogenates. As shown in **Fig 4.8c**, IL-10 expression profiles in A/PR/8 and A/WSN/33 infected lungs were identical and peaked at day 7 post-infection suggesting that both highly virulent and less virulent strains of influenza do induce pulmonary IL-10 expression.

Figure 4.8 Mortality and weight loss following infection with influenza A/WSN/33

a.



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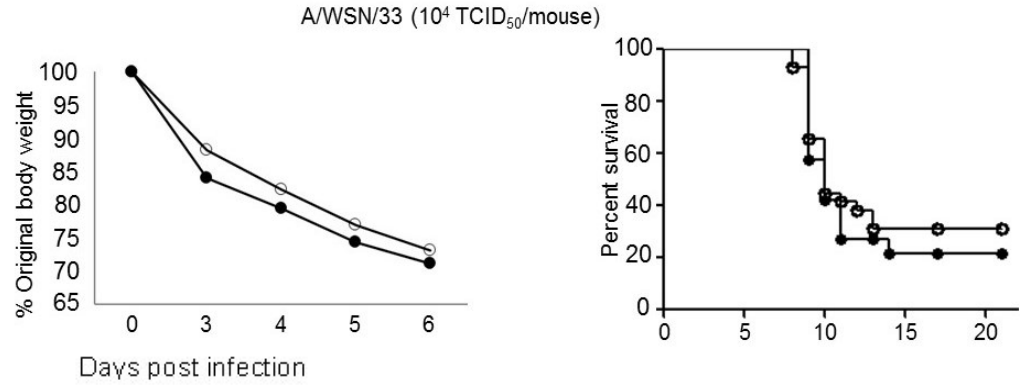

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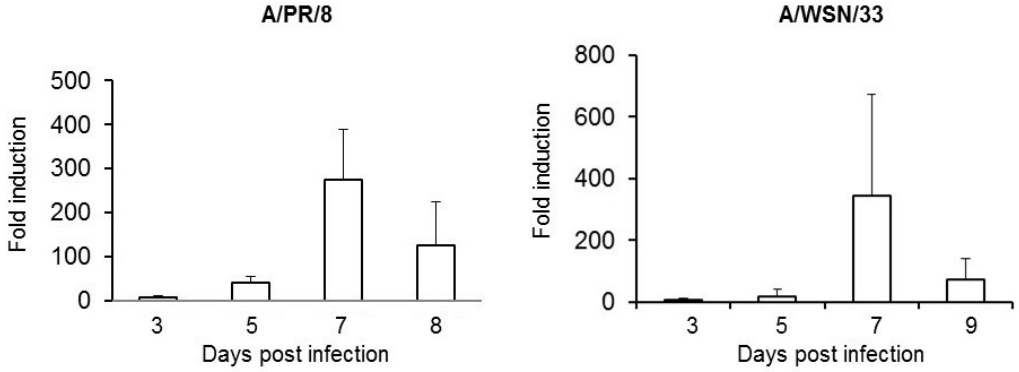

Fig 4.7. *Il10*$^{-/-}$ and WT mice were infected with (**a**) 10^3 $TICD_{50}$/mouse and (**b**) 10^4 $TICD_{50}$/mouse of A/WSN/33 and monitored over a period of 21 days for survival and weight loss (n=30 mice/group) (**c**) WT mice infected with A/PR/8 or A/WSN/33 and on the stated days lungs were analyzed for IL-10 gene expression by RT-PCR (n=7-10 mice/group/time point. Data combined from 2 independent experiments).

4.5 Discussion

Influenza virus pneumonia is characterized by the rapid development of pulmonary injury and is typically associated with poor disease outcomes (34,35). Both viral pathogenicity factors and a dysregulated immune response have been implicated in the induction of pulmonary failure (53,305,306). However, the immunological mechanisms that govern the development of pulmonary injury are poorly understood. Therefore in this study, we examined the role of the anti-inflammatory cytokine IL-10 in regulating immunopathology during influenza infections.

The primary function of IL-10 is in modulating the expression of cytokines and chemokine such as TNF-α, IL-6, IL-1β, CCL-2 and IL-8 as well as down regulating the expression of co-stimulatory molecules on APC's, thereby affecting their ability to initiate and sustain an inflammatory response (reviewed in (283)). By doing so, IL-10 has also been shown to have a profound impact on the development of $T_{effector}$ subsets including Th1, Th2 and Th17 cells (307,308). In keeping with its canonical functions, we too found that expression of IL-10 during influenza infections served to regulate the expression of several key proinflammatory cytokines and recruitment of $T_{effector}$ populations into pulmonary tissue. Interestingly, while we and others (112) did not detect any IL-17 protein in the BALF of WT or *Il10*$^{-/-}$ mice (data not shown) nor any increase in IL-17 producing CD4^{+} or CD8^{+} T cells (data no shown), *McKinstry*, et al., noted significant increases in IL-17 producing CD4 T cells in the lungs of *Il10*$^{-/-}$ compared to WT. One potential explanation for these conflicting results is that different strains of mice were used, ie. we used C57Bl/6 mice, whereas McKinstry, et al. utilized BALB/c mice. Indeed other studies have similarly noted mouse strain-specific expression patterns of IL-17 (309). Nevertheless IL-17 production in lung tissue or the recruitment of other cell types capable of making IL-17 cannot be ruled out.

During chronic infections, the potent anti-inflammatory activity of IL-10 has been shown to interfere with effective clearance of pathogens such as *Leishmania* (292), lymphocytic choriomeningitis virus (LCMV)(293) and *M. tuberculosis* (310). However, during acute influenza infection, we observed no negative effect of IL-10 on viral replication. Similarly IL-10 had no effect on viral replication in a mouse model of respiratory syncytial virus (RSV) (311). Taken together these data suggest that, at least in the context of acute respiratory viral infection, the regulatory effects of IL-10 may not significantly impact the development of anti-viral effectors.

The role of IL-10 in influenza has been somewhat controversial: While Sun J, et al., noted significantly lower survival rates in mice treated with IL-10R blocking antibodies 3 days post-infection, McKinstry KK., et al. observed significantly higher survival rates when antibody administration was started prior to infection or when IL-10 was genetically ablated (ie. *Il10*$^{-/-}$), similar to findings in this study (110,111). These conflicting data suggest that perhaps the temporal expression and therefore the cellular source of IL-10 may be key in dictating disease susceptibility. IL-10 is a pleiotropic cytokine that is secreted by many different cell types including macrophages, CD4 and CD8 T cells, B cells and epithelial cells (283). In the context of influenza, while some studies have demonstrated that pulmonary CD4 and CD8 T cells are an important source of IL-10 (110,111), others report significant IL-10 expression even after T cell depletion indicating the presence of other sources of IL-10 (112). It will be interesting in future studies to more rigorously determine the requirement for specific cellular sources of IL-10 in influencing disease susceptibility to influenza infections using cell type-specific knock out strategies such as the Cre/loxP system.

During influenza infections, virus replication and the presence of inflammatory mediators has been shown to result in the apoptosis of pulmonary epithelial cells thereby

leading to substantial tissue damage and decreased pulmonary function (304). At day 8 post-infection, while there were signs of active apoptosis in WT lungs, *Il10*$^{-/-}$ lungs appeared to have cleared most apoptotic cells, suggesting a possible acceleration of tissue repair/reduction of pulmonary injury in IL-10 deficient lungs. In agreement with this, another study found that IL-10 inhibited wound closure and healing in cutaneous model of injury by inhibiting the activation of (312).

Repair and remodeling of pulmonary tissue after damage is a complex process that involves the coordinated action of cytokines, chemokines, growth factors and extracellular matrix proteins (reviewed in (313)). During the repair process, local and newly recruited progenitor cells undergo proliferation, differentiation and migration to re-establish the denuded epithelial layer. Members of the epidermal growth factor (EGF) (314) (315) and fibroblast growth factor (FGF) families (reviewed in (316)) have long been known to be involved in this process. However, a role for inflammatory mediators in tissue repair and homeostasis has also been proposed. IL-22, a member of the IL-10 family, has recently emerged as an important player in the maintenance of tissue homeostasis. IL-22 can act directly on epithelial cells and induce the expression of gene involved in wound healing via STAT3 signaling (317). Consequently, IL-22 has been shown to promote mucosal tissue repair during influenza infections (115,303). Despite a significant up regulation of IL-22 in *Il10*$^{-/-}$, as previously reported (111), our data indicate the protection in *Il10*$^{-/-}$ does not depend on IL-22. Further studies will thus be required to determine the specific mechanisms responsible for decreased/delayed pulmonary injury noted in *Il10*$^{-/-}$ mice but it is tempting to speculate that regulation of immune or non-immune mechanisms of tissue repair are involved. For instance, IL-1β has been shown to augment alveolar epithelial migration and cell spreading by inducing the expression of EGF or TGF-α in an *in vitro* model (318,319). Similarly innate lymphoid cell recruitment

into influenza-infected lungs has been shown to be critical in promoting airway epithelial cell integrity and lung function via the secretion of amphiregulin (116) (a member of the EGF family (320)). The regulation of these effectors by IL-10 is not well characterized and warrants further study.

One intriguing finding in this study was the previously unreported virus-specific effect of IL-10 on disease outcome. Influenza viruses can differ remarkably in their ability cause cell death and pulmonary tissue destruction, with highly virulent strains generally resulting in significantly greater pathology. This difference in pulmonary damage can be driven by differential ability of influenza viruses to directly cause apoptosis in infected cells (321). Indeed, inserting the NA and PA gene segments from A/PR/8 into less virulent strains was found to dramatically increase the levels of apoptosis in cultured MDCK cells *in vitro* (322). Alternatively, influenza viruses can differ in their ability to recruit and/or activate inflammatory cells, thereby indirectly causing increased pulmonary damage (89,237,304). In light of our data showing continued presence of apoptotic cells in WT compared to *Il10*$^{-/-}$ mice, one could speculate that the suppression of tissue repair/enhancement of tissue injury by IL-10 dependent mechanisms may more directly impact disease outcomes with highly virulent strains such as PR/8 compared to less virulent infections. Further studies are required to elucidate the mechanism behind this finding.

CHAPTER 5: General discussion

Despite vaccination efforts, influenza remains one of the leading causes of death in the United States (http://www.cdc.gov/nchs/data/nvsr/nvsr61/nvsr61_06.pdf). A major hurdle in the development of new therapeutic approaches and vaccines is our limited understanding of host factors that contribute to protective and pathogenic responses to infection. While the majority of past work has focused on neutralizing antibodies as the sole correlate of protection against infection, emerging data suggest that other components of the immune system may not only play an important role in protective immunity but, in fact, could also cause substantial tissue damage. The work presented in this dissertation contributes significantly to the better understanding of immune mechanisms that contribute to protective immunity during vaccination and pathologic host responses during lethal influenza infections *in vivo*.

Vaccines are widely regarded as one of the most successful public health interventions to date, with a 95-99% reduction in the incidence of vaccine preventable diseases in the United States (323). In recent years there has been an unprecedented development of new vaccine strategies, nevertheless, the development of a “universal influenza vaccine” or even more generally, successful vaccines against HIV, malaria and tuberculosis has been slow and challenging. This is, in part, because we still do not fully understand effector mechanisms that best protect against or eliminate these pathogens, particularly in humans (324,325). Neutralizing antibodies have been the cornerstone of influenza vaccine efforts for decades. Only recently have we begun to understand the importance of T cell immunity in generating long-lasting, cross-reactive immunity to influenza (68,78,81,263). Understandably, there are still a number of unanswered questions. For instance: what effector functions (the ability to make cytokines,

chemokines or cytotoxic molecules) should antigen-specific CD4 and CD8 T cells express to mediate optimum protection? What state of differentiation (effectors, central memory or effector memory) should they be in at the time of infection? How many of the proper type of cell is required to mediate optimum immunity? Which epitope(s) should the T cell and B cell responses be directed against to ensure maximum effectiveness against different strains? What type of formulation (live, vectored, inactivated, etc.) and by what route (intranasal, intramuscular, intradermal, etc.) should the vaccine be administered to maximally stimulate this protective response? Are there immunological changes that occur soon after vaccination that can predict the development of protective immunity so as to facilitate the early identification of non-responders?

Some of the above mentioned questions regarding the development of adaptive immune responses have been described in animal models, as briefly outlined in section 1.2. However, despite their similarities, differences in the immune system between mice and humans makes the direct translation of these findings in mice to human disease settings challenging and time-consuming. Ethical and practical considerations limit our ability to perform similar studies in humans. However, vaccination represents a unique system that allows us do this. Obtaining blood samples at various time points post-vaccination allows us to study the exact kinetics of innate and adaptive responses in humans and yet this is an opportunity that has been largely unexploited. As a part of this dissertation, we used seasonal influenza vaccines as a model to gain insight into adaptive immune mechanisms (Chapters 2 and 3).

In chapter 2, I described the serum antibody response to seasonal TIV and LAIV. As has been previously been shown, we too observed robust increases in strain-specific serum antibody titers following parenteral vaccination with TIV. Studies in mice suggest that the development of primary and memory B cell responses requires a highly

coordinated interaction between cognate B cells and specialized follicular helper T cell (T_{FH}) in germinal centers (67). In addition to providing cell associated signals, T_{FH} cells secrete a variety of cytokines including IL-4, IFN-γ and IL-21 which are thought to be crucial to class switching and antibody production. While such molecular events are readily discernible in mouse models, they are near impossible to characterize in humans. In this study, we measured cytokines in the serum as possible biomarkers of such immunological events (Chapter 2). At the peak of the antibody response (day 14 post-vaccination), we were able to detect significant changes in two important pro-inflammatory cytokines IL-8 and TNF-α. Measurement of serum cytokines is relatively easy, cost effective and can be useful biomarkers of "vaccine take" or even adverse reactions. The data presented in this dissertation serve as proof of principle that serum cytokine changes following vaccination can be readily detected in individuals. Future studies aimed at identifying early changes in serum cytokines that correlate with or predict later immunogenicity (antibody production, etc) and/or protection after vaccination can help in the early identification of non-responders, which is of great interest from a public health standpoint.

The lack of understanding of T cell immunity in humans has, at least in part, been due to the lack of suitable laboratory techniques to reliably and consistently enumerate antigen-specific T cells. The added variability in their functional capacity to make effector molecules as well as their surface phenotype has compounded the complexity of T cell measurements. In chapter 3, we describe a novel flow cytometry-based platform for the functional and phenotypic characterization of antigen-specific T cells. Unlike soluble assays, the batch lyophilization process ensures consistency of reagents across multiple days and possibly even laboratories thereby minimizing day-day variability (225). We believe that this study may be an important step in the generation of standardized

reagents for the large-scale assessment of cellular immunity against not only influenza but also other agents.

The limited efficacy of the seasonal vaccines in various settings (as outlined in section 1.3.2) and the current difficulty in generating successful new vaccines emphasizes the need to base future vaccine design strategies on an in-depth understanding of effector responses that are most beneficial for protective immunity and an understanding of how best to stimulate such responses (325). As the landscape of influenza vaccines evolves over the next few years, the development of high-throughput technologies and systems biology efforts that allow for the rapid and in-depth measurement of such responses is likely to accelerate the pace of vaccine development in the future (324,326). We believe the work described in this thesis takes an important step in that direction. We have utilized and developed innovative technologies to more comprehensively evaluate the humoral and cellular immune response to seasonal influenza vaccines. We have described our unexpected finding of serum cytokines as potential biomarkers of influenza vaccine induced immunity as well as the previously unappreciated impact of vaccination on cellular immune responses. However, several unanswered questions remain. Do the changes in immune markers we observed correlate with protection from infection/ severe disease? Will measurements of cytokines or antibodies in nasal wash or bronchial lavage samples be more indicative of immunological changes following LAIV? Can such changes be used to predict adverse events following vaccination?

While the focus of the work presented here has been on adaptive immunity, in recent years, there has been an increased appreciation of the key role the innate immune system plays in sensing pathogens/vaccines/adjuvants and shaping the magnitude of the adaptive immune response (327). Several phenotypically distinct subsets of

immature and mature dendritic cells have been identified in both mice and human blood (reviewed in (328)). These subsets are thought to differ in their microenvironmental localization, TLR expression and function, although there is also evidence of some degree of plasticity between them. As we gain more insight to the kind of immune responses that are most effective in controlling specific pathogens, understanding how to manipulate innate immune responses using different adjuvants and/or delivery routes so as to generate the appropriate adaptive immune response and long-lived memory is key.

In the final chapter of this thesis (Chapter 4) we sought to understand mechanisms that govern the development of pulmonary pathology during lethal influenza infection *in vivo*. In humans, severe disease caused by influenza is thought to be, in part, mediated by excessive inflammation resulting in severe pulmonary immunopathology (89). Due to poor disease outcomes in patients, particularly in young children and older adults (>65 years of age), there has been a lot of interest in developing adjunct immunomodulatory therapies than can be used in combination with anti-virals. However, exact mechanisms that contribute to uncontrolled inflammation are not clear. IL-10 is a potent anti-inflammatory cytokine that is known to play a key role in mediating immune homeostasis in numerous infectious and autoimmune diseases. Therefore, in chapter 4, we explored the role of IL-10 in controlling pulmonary immunopathology.

Influenza virus pneumonia often occurs with or is followed by secondary bacterial pneumonia. In fact, approximately 96% of all deaths during the 1918 pandemic were likely due to secondary bacterial infections (113). Interestingly, IL-10 expression during influenza infection has been linked with increased susceptibility to secondary bacterial infections (329). In this study, we found that IL-10 regulated the expression of pulmonary IL-22. In addition to contributing to tissue repair, IL-22 has also been shown to play an

important role in controlling bacterial load by up-regulating the expression of antimicrobial proteins in pulmonary epithelial cells (330). It will therefore be interesting to utilize the *Il10*$^{-/-}$*Il22*$^{-/-}$ generated in this study to determine if increased expression of IL-22 in *Il10*$^{-/-}$ has an impact on secondary bacterial infection outcomes.

The translation of findings in animal models to human disease represents a major challenge in identifying targets for intervention. Several recent studies have shown an association between IL-10 expression in serum and disease severity during influenza infections in humans, lending support to our findings (331,332). However, these studies do not reveal the cellular source of human IL-10 nor its function *in vivo*. A novel approach taken by *Ranatunga*, et al. utilizes BAC transgenic animals that carry the gene of interest along with most if not all the regulatory elements required for cell-type specific expression of the gene *in vivo* (Ranatunga, et al. PNAS). In future studies, it will be of interest to use this approach to gain insight into the role of human IL-10 during severe influenza infections.

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CURRICULUM VITAE

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EDUCATION

2006-present	Ph.D. (Expected October 2013), Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, MD
2008-2009	Certificate of Vaccine Science and Policy, Johns Hopkins School of Public Health, Baltimore, MD
2002-2006	Bachelor of Technology, Department of Biotechnology, Anna University, Chennai, India

RESEARCH EXPERIENCE

Graduate student (August 2006-May 2008 Sc.M. student; 2008-present Ph.D. candidate)

Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, MD

Mentor: Jay Bream, Ph.D.

Principle responsibilities: Conduct independent graduate level research

- Characterized the immune response to seasonal live attenuated (LAIV) and inactivated (TIV) influenza vaccines in a human cohort to identify potential correlates of protection against pandemic influenza
    - Developed a high-throughput, 10 color flow cytometry assay to perform functional and phenotypic characterization of influenza-specific T cells
    - Evaluated the impact of seasonal vaccination on cross-reactive antibody responses
    - Profiled serum cytokines as innovative biomarkers of antibody responses and adverse reactions
- Demonstrated an unexpected pathogenic role for IL-10 in influenza induced pulmonary injury
    - Developed a mouse model of lethal influenza infection
    - Generated and maintained 6 transgenic and conditional gene knock out mouse lines
    - Quantified pulmonary inflammation by cytokine expression profiling, flow cytometric quantification of infiltrating leukocyte populations, histopathological examination of pulmonary tissue and viral load measurement

- Collaborated extensively on other laboratory projects leading to 2 co-author publications
    - Assisted in defining methylation patterns of the mouse and human *IL10* genes during $CD4^+$ T cell activation.
    - Assisted in characterizing the *in vivo* role of $CD4^+$ T cell-derived human IL-10 in mediating protection from colitis utilizing a novel transgenic hIL10BAC mouse model

RELEVANT INDUSTRY EXPERIENCE

Intern (June 2011-December 2012)

Acidophil, LLC, Baltimore MD

Principle responsibilities: Aid in the identification of new business/technology opportunities

- Evaluated intellectual property using Delphion, USPTO and Google Patent Search
- Analyzed the competitive landscape and technical feasibility of various biotech products
- Conducted relevant market research analysis using databases such as Frost and Sullivan
- Drafted technical reports including competitive grant applications and due diligence summaries

PUBLICATIONS

- **Ramakrishnan A**, Althoff KN, Lopez JA, Coles CL and Bream JH. Differential serum cytokine responses to inactivated and live attenuated seasonal influenza vaccines. *Cytokine* 2012. 60(3):661-6.

- Ranatunga DC, **Ramakrishnan A,** Uprety P, Wang F, Zhang H, Margolick JB, Brayton C Bream JH. A protective role for human IL-10 expressing $CD4^+$ T Cells in colitis. *J Immunol*. 2012. 189(3):1243-52.
- Hedrich CM, **Ramakrishnan A**, Dabitao D, Ranatunga DC, Bream JH. 2010. Dynamic DNA methylation patterns across the mouse and human *IL10* genes during $CD4^+$ T cell activation; influence of IL-27. *Mol Immunol.*2010.48 (1-3):73-81.

In preparation

- **Ramakrishnan A**, Inokuma M, Pekosz AS, Jacobson LP, Maino VC and Bream JH. Heterotypic T cell responses to inactivated and live attenuated seasonal influenza vaccines.
- **Ramakrishnan A,** Ranatunga DC, Mitzner W, Brayton C, Bream JH. A pathogenic role for IL-10 in the development of pulmonary injury during lethal influenza infection.

SCIENTIFIC PRESENTATIONS

Invited oral presentations

1. **Ramakrishnan A** and Bream JH. 2013. Immune profiling of seasonal influenza vaccines. Oral presentation at the Naval Medical Research Unit 6 (NAMRU-6), Lima, Peru.
2. **Ramakrishnan A** and Bream JH. 2012. Immune profiling of seasonal influenza vaccines. Oral presentation at the Molecular Microbiology and Immunology departmental retreat, Johns Hopkins School of Public Health, Baltimore, MD.

Poster presentations

1. **Ramakrishnan A**, Inokuma M, Maino VC, Pekosz AS and Bream JH. 2012. Immune profiling of seasonal influenza vaccines. Poster presentation at the annual Johns Hopkins Vaccine Day, Baltimore, MD
2. **Ramakrishnan A**, Inokuma M, Maino VC, Pekosz AS and Bream JH. 2011. The cellular immune response to seasonal influenza vaccines. Poster presentation at the annual Johns Hopkins Vaccine Day, Baltimore, MD.
3. **Ramakrishnan A**, Althoff KN, Pekosz AS and Bream JH. 2010. Immune response to seasonal influenza vaccination. Poster presentation at the 97th Annual Meeting of the American Association of Immunologists, Baltimore, MD.
4. **Ramakrishnan A**, Althoff KN, Pekosz AS and Bream JH. 2009. Immune response to seasonal influenza vaccination. Poster presentation at the annual Johns Hopkins Vaccine Day, Baltimore, MD.
5. **Ramakrishnan A**, Dabitao D, Ranatunga DC, Hedrich CM, Wang FY and Bream JH. 2008. Serum cytokine profiles following seasonal influenza vaccination. Poster presentation at the annual Johns Hopkins Delta Omega Honor Society, Baltimore, MD.

LABORATORY SKILLS

Multiparameter flow cytometry

- Extensive experience with multicolor flow cytometry (10 colors)
- Antibody panel design, assay development and optimization for phenotypic and functional characterization of antigen specific T cells
- Flow cytometry data acquisition using BD LSRII and BD FACSCalibur and data analysis using BD FACSDiva and FlowJo (Tree Star)

Molecular biology techniques

- RNA and DNA extraction, cDNA synthesis, standard PCR and real-time PCR using SYBR green and TaqMan assays
- Designing primers for use in SYBR green and TaqMan assays
- ELISA and multiplex cytokine analysis from cell culture supernatant, mouse tissue and mouse and human sera using BD CBA, FlowCytomix (eBioscience) and Meso Scale Discovery (MSD)
- Immunohistochemistry and TUNEL staining

Cell culture

- Human PBMC isolation and antigen specific stimulation using overlapping peptide pools and live and inactivated viruses for use in flow cytometry assays
- Aseptic cell culture techniques
- Propagation and *in vitro* stimulation of the following cell lines: Madin-Darby Canine Kidney (MDCK) epithelial cells, RAW 264.7 mouse monocyte/macrophage cell line, L929 mouse fibroblasts and A549 human alveolar epithelial cells
- Generation of mouse bone marrow derived macrophages and dendritic cells

Animal experience

- *In vivo* infection of mice (influenza)
- Intranasal, intramuscular, intratracheal and intraperitoneal delivery in mice
- Isolating mouse lungs, spleens, bronchoalveolar lavage fluid (BALF), intraepithelial lymphocytes (IEL), lamina propria lymphocytes (LPL) and mediastinal and mesenteric lymph nodes
- Processing mouse tissue for histological examination, cytokine/chemokine quantification, flow cytometric examination of leukocyte populations, Fluorescence Activated Cell Sorting (FACS) and/or gene expression by real-time

- Animal husbandry and maintenance of large mouse colonies (6 independent transgenic lines with a total of 300-400 animals), tail snip genotyping, breeding and backcrossing

Virology techniques

- Virus propagation *in vitro* (human isolates of influenza, mouse adapted influenza strains and Vesicular Stomatitis Virus)
- Titration of virus from cell culture supernatant and mouse lungs using plaque assay, real-time PCR, standard hemagglutination assay and 50% Tissue Culture Infective Dose ($TCID_{50}$)
- Influenza specific antibody titration using hemagglutination inhibition assay

- Statistical analysis using Microsoft Excel, Prism (GraphPad) and SPICE

HONORS AND AWARDS

2010 Delta Omega Scholarship ($1,000), Johns Hopkins School of Public Health, MD

2009 Eleanor A. Bliss Honorary Fellowship, Johns Hopkins School of Public Health, MD

2008 Edward and Kathy Ludwig Fellowship ($50,000), Johns Hopkins School of Public Health, MD

2007 Master's Tuition Scholarship ($25,000), Johns Hopkins School of Public Health, MD

TEACHING EXPERIENCE

Teaching assistant (2007- present)

Johns Hopkins School of Public Health, MD

Principle responsibilities: Organize small group discussions, tutor and assist students and provide administrative assistance for the following graduate level courses

- Introduction to Biomedical Sciences (2007)
- Graduate Immunology (2008 and 2013)
- Biological Basis of Ageing (2008 and 2009)
- Biological Basis of Vaccine Development (2009)